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INTERPLAY BETWEEN THE RENIN-ANGIOTENSIN SYSTEM AND VASCULAR SYMPATHETIC NEUROTRANSMISSION IN FETAL PROGRAMMING OF HYPERTENSION: A FOCUS ON ADVENTITIA LAYER.

TESIS DOCTORAL

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CERTIFICAN

Que Dña Maria Sofia Vieira da Rocha ha realizado bajo su dirección el trabajo: "**Interplay between the renin-angiotensin system and vascular sympathetic neurotransmission in fetal programming of hypertension: a focus on adventitia layer**." con objeto de obtener el Grado de Doctor.

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Adapt yourself to the environment in which your lot has been cast, and show true love to the fellow-mortals with whom destiny has surrounded you.

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INDEX

Hypothesis and Aims

Materials and Methods

Results

FIGURES INDEX

TABLES INDEX

ABBREVIATIONS

$\bullet O_2^-$	Superoxide anion
11β-HSD2	11 β-hydroxysteroid dehydrogenase type 2
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
Ang 1-7	Angiotensin (1-7)
Ang II	Angiotensin II
ATP	Adenosine triphosphate
BP	Blood pressure
COX	Cyclooxygenase
CVDs	Cardiovascular diseases
DBP	Diastolic blood pressure
EDCF	Endothelium-derived contracting factors
EDRF	Endothelium-derived relaxing factors
ELISA	Enzyme-Linked Immune Sorbent Assay
eNOS	Endothelial nitric oxide synthase
FPH	Fetal programming oh hypertension
GC	Glucocorticoids
GFAP	Glial fibrillary acidic protein
GPCRs	G-protein coupled receptors
H_2O_2	Hydrogen peroxide
HR	Heart rate
IL-1	Interleukin 1
KHS	Krebs–Henseleit solution
LBW	Low birth weight
LVDD	Left ventricular diastolic dysfunction
LVH	Left ventricular hypertrophy
MAP	Mitogen-activated protein
MMP	Matrix metalloproteinases
MrgD	Mas-related G protein coupled receptor D
Mrgs	Mas-related genes

MUN	Offspring exposed to maternal undernutrition
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NO	Nitric oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate buffer solution
RAS	Renin-Angiotensin system
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SMC	Smooth muscle cells
SNA	Sympathetic nerve activity
SNS	Sympathetic nervous system
TGF-β	Transforming growth factor-beta
TH	Tyrosine hydroxylase
UAM	Universidad Autónoma de Madrid
VSMC	Vascular smooth muscle cells
WHO	World Health Organization
WKY	Wistar-Kyoto rats

Abstract

Resumen

ABSTRACT

Background. Cardiovascular diseases (CVDs) are the main cause of morbidity and mortality due to non-communicable diseases worldwide. Together with the already known CVDs risk factors related to lifestyle and genetic background, it is well recognized the importance of fetal life. The fetus can adapt to adverse intrauterine conditions to ensure survival. However, these conditions induce alterations in fetal development, which result, later in life, in an increased susceptibility to develop CVDs and its risk factors, particularly hypertension. This is known as Fetal Programming of Hypertension (FPH). Despite the efforts carried out so far, the mechanisms underlying the predisposition to FPH are not completely understood although some have been proposed to explain the relationship between poor fetal growth and later hypertension development. Two of the proposed mechanisms are the alteration of the renin-angiotensin system (RAS) and an activation of sympathetic nervous system (SNS). However, the possible relationship between these two systems and their contribution to vascular functional and structural alterations in FPH is still to be addressed.

In this Thesis the following **hypothesis** is postulated: "suboptimal nutrition during fetal life induces hypertension in male rats, at least in part, through an alteration in vascular sympathetic neurotransmission, and cross-talk with RAS". The **aim** of this Thesis was to evaluate in a rat model of FPH if the vascular SNS and the RAS are altered, their interplay in vascular sympathetic neuromodulation, and contribution to arterial remodelling. Additionally, the SNS, vascular remodelling and hemodynamic alterations have been compared to those found in spontaneously hypertensive rat (SHR), a rat model of essential hypertension. The specific aims were to evaluate in male rats the influence of fetal undernutrition on: 1) hemodynamic parameters, mesenteric artery remodelling and sympathetic neurotransmission, comparing with SHR model, and 2) RAS alterations, assessing vascular expression of enzymes and receptors, circulating levels of angiotensin II (Ang II) and effect on sympathetic neurotransmission. **Methods**. This study was conducted in offspring from an experimental model of fetal programming in Sprague Dawley rats, induced by 50% restriction of maternal intake during the second half of pregnancy (Maternal Undernutrition, MUN), using as control, offspring from rats fed *ad libitum* (CONTROL). SHR rats were used as rat model of essential hypertension, being

control the Wistar Kyoto (WKY) rat. The following parameters were assessed in 6 month old male offspring: 1) hemodynamic parameters (intra-arterial measurements in anesthetized rats and tail-cuff plethysmography); 2) arterial remodelling (histomorphometric analysis of mesenteric artery, MA); 3) sympathetic innervation (fluorescence immunohistochemistry in MA); 4) sympathetic neurotransmission and the neuromodulatory role of Ang II (^{3}H-noradrenaline release by electrical field stimulation); 5) plasma determination of Ang II (ELISA); 6) expression of RAS enzymes (renin, angiotensin converting enzyme (ACE) and ACE2) and receptors (AT_1, AT_2, Mas and MrgD) by RT-PCR in MA; 7) RAS receptors distribution in the adventitia of MA (fluorescence immunohistochemistry); 8) Localization of NADPH oxidase, the main enzyme responsible for ROS production, in the adventitia of MA (fluorescence immunohistochemistry) . **Results**. Fetal nutrient restriction induces: 1) hypertension; 2) inward hypertrophic remodelling with fibrosis of MA; 3) higher immunoreactivity for tyrosine hydroxylase (sympathetic nerves marker); 4) higher noradrenaline release and uptake; 5) higher tonic facilitation by endogenous Ang II through presynaptic AT_1 receptors; 6) higher RNA expression of ACE1 and ACE2; 7) higher RNA expression of AT_1 receptors and lower levels of AT_2, Mas and MrgD receptors; 7) higher immunoreactivity for NADPH oxidase in the adventitia layer. **Conclusions.** Adult males exposed to fetal undernutrition exhibit vascular sympathetic innervation hypertrophy and hyperactivation of sympathetic neurotransmission, at least in part, related to facilitation by Ang II through AT_1 receptors. These modifications may be responsible for the observed remodelling and fibrosis through oxidative damage. All of these findings contribute to increase vascular resistance and consequently, to hypertension. The sympathetic alterations and vascular remodelling alterations found in FPH are similar to those in SHR.

RESUMEN

Antecedentes. Las enfermedades cardiovasculares (ECV) son la principal causa de morbilidad y mortalidad por enfermedades no transmisibles en el mundo. Junto con los factores de riesgo clásicos relacionados con el estilo de vida y antecedentes genéticos, se conoce la importancia de la vida fetal en el desarrollo de ECV. Ante un ambiente intrauterino adverso, el feto se adapta para asegurar la supervivencia. Sin embargo, estas condiciones inducen alteraciones en su desarrollo, lo que conduce a una mayor susceptibilidad para desarrollar ECV y sus factores de riesgo -particularmente hipertensión- en etapas posteriores de la vida. Esto se conoce como programación fetal de la hipertensión (FPH). A pesar de los esfuerzos, los mecanismos subyacentes a la programación fetal no se comprenden completamente, aunque se han propuesto algunos para explicar la relación entre un crecimiento fetal deficiente y el desarrollo posterior de hipertensión. Dos de los mecanismos propuestos son la alteración del sistema renina-angiotensina (SRA) y la activación del sistema nervioso simpático (SNS). Sin embargo, la posible relación entre estos dos sistemas y su contribución a las alteraciones vasculares funcionales y estructurales en la FPH aún no se ha abordado. En esta Tesis se postula la siguiente **hipótesis**: "la nutrición subóptima durante la vida fetal induce hipertensión en ratas machos, al menos en parte, a través de una alteración en la neurotransmisión simpática vascular, a través de una interacción con el SRA". El **objetivo** de esta Tesis fue evaluar en un modelo de FPH en rata si el SNS vascular y el SRA están alterado, si existe interacción entre ambos sistemas en la neuromodulación simpática vascular y la contribución de estas alteraciones al remodelado vascular. Además, las alteraciones hemodinámicas, el remodelado vascular y las modificaciones del SNS se han comparado con las encontradas en ratas espontáneamente hipertensas (SHR), un modelo de rata de hipertensión esencial. Los objetivos específicos fueron evaluar en ratas machos la influencia de la desnutrición fetal en: 1) parámetros hemodinámicos, el remodelado y la neurotransmisión simpática de la arteria mesentérica (AM), comparando los resultados con el modelo SHR de hipertensión esencial y 2) las alteraciones en el SRA, evaluando la expresión de enzimas y receptores, niveles circulantes de angiotensina II (Ang II) y su posible efecto sobre la neurotransmisión simpática. **Métodos.** Este estudio se realizó en descendientes de un

modelo experimental de programación fetal, en ratas Sprague Dawley, inducida por una restricción del 50% de la ingesta materna durante la segunda mitad de la gestación (Maternal Undernutrition, MUN), utilizando como control, descendientes de ratas alimentadas *ad libitum* (CONTROL). Las ratas SHR se utilizaron como modelo de rata de hipertensión esencial, siendo su control la rata Wistar Kyoto (WKY). Los siguientes parámetros se evaluaron en descendientes macho de 6 meses de edad: 1) parámetros hemodinámicos (medida intra-arterial en ratas anestesiadas y pletismografía en la cola; 2) remodelado vascular (análisis histomorfométrico de AM) ; 3) inervación simpática (inmunohistoquímica de fluorescencia y microscopia Confocal en AM); 4) neurotransmisión simpática en la AM y el papel neuromodulador de Ang II (experimentos de liberación de ^{3}H-noradrenalina por estimulación eléctrica); 5) determinación plasmática de Ang II (ELISA); 6) expresión de enzimas del SRA (renina, enzima convertidora de angiotensina (ACE1) y ACE2) y receptores (AT_1, AT_2, Mas y MrgD) por RT-PCR en AM; 7) distribución de receptores del SRA en la adventicia arteria mesentérica (immunohistoquímica de fluorescencia); 8) la presencia de NADPH oxidasa, la enzima principal responsable de la producción de ROS, en la adventicia de la AM (immunohistoquímica de fluorescencia y microscopia Confocal). **Resultados**. La restricción de nutrientes en la etapa fetal induce: 1) hipertensión; 2) remodelado hipertrófico de tipo “inward” con fibrosis en la AM; 3) mayor immuno-reactividad para tirosina hidroxilasa (marcador de inervación simpática); 4) mayor liberación y absorción de noradrenalina; 5) facilitación tónica por Ang II endógena a través de receptores presinápticos AT_1; 6) mayor expresión de ARN de las enzimas ACE1 y ACE2; 7) mayor expresión de ARN de los receptores AT_1 y menor de los receptores AT_2, Mas y MrgD; 7) mayor immunoreactividad para NADPH oxidasa en la capa de adventicia. **Conclusiones**. En la etapa adulta, los machos expuestos a desnutrición fetal muestran una hipertrofia de la inervación simpática vascular e hiperactivación de la neurotransmisión simpática, al menos en parte, relacionada con alteraciones del SRA vascular. Estas modificaciones pueden ser responsables del remodelado y la fibrosis vascular a través de daño oxidativo. Todas estas alteraciones contribuyen a aumentar la resistencia vascular y, en consecuencia, contribuir al desarrollo de hipertensión. Las alteraciones del SNS y del remodelado vascular encontradas en FPH, son similares a las encontradas en ratas SHR.

Introduction

I. CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) are considered non-communicable chronic diseases and are the result of a combination of genetic, physiological, environmental and behaviours factors. CVDs comprise disorders of the heart and of blood vessels, including those supplying the heart muscle, the brain, the kidney, the arms and legs, among others. The most important acute cardiovascular events occur mainly because a blockage that prevents blood from flowing to the heart or brain. In the heart, atherosclerosis or abnormal blood clots, can lead to a thrombus and cut off or reduce the blood flow to part of the heart muscle. This is known as Coronary Heart Disease. When the blood flow to the heart is cut off, the decrease in oxygen and nutrients supply damages the heart tissue resulting in a heart attack. In the brain, a reduction of blood flow can lead to a stroke. A stroke can be ischemic when a blood vessel that irrigates the brain gets blocked, usually from a blood clot or atherosclerosis, or hemorrhagic, when a blood vessel within the brain bursts. Hemorrhagic stroke is most often caused by uncontrolled hypertension. In addition to acute cardiovascular events, CVDs also lead to chronic alterations, like Congestive Heart Failure (reduced pumping capacity of the heart), atrial fibrillation (uncoordinated atrial activation) and Chronic Kidney Failure (reduced capacity of the kidneys to carry out their function). High blood pressure (BP) also plays a key role in the development of these chronic CVDs [1].

i. EPIDEMIOLOGY OF CARDIOVASCULAR DISEASES

CVDs are the number one cause of morbidity and mortality in the world, and are responsible for 49% of mortality in Europe, being ischemic heart disease and stroke the main events responsible for the higher percentage of deaths (Figure 1) [2].

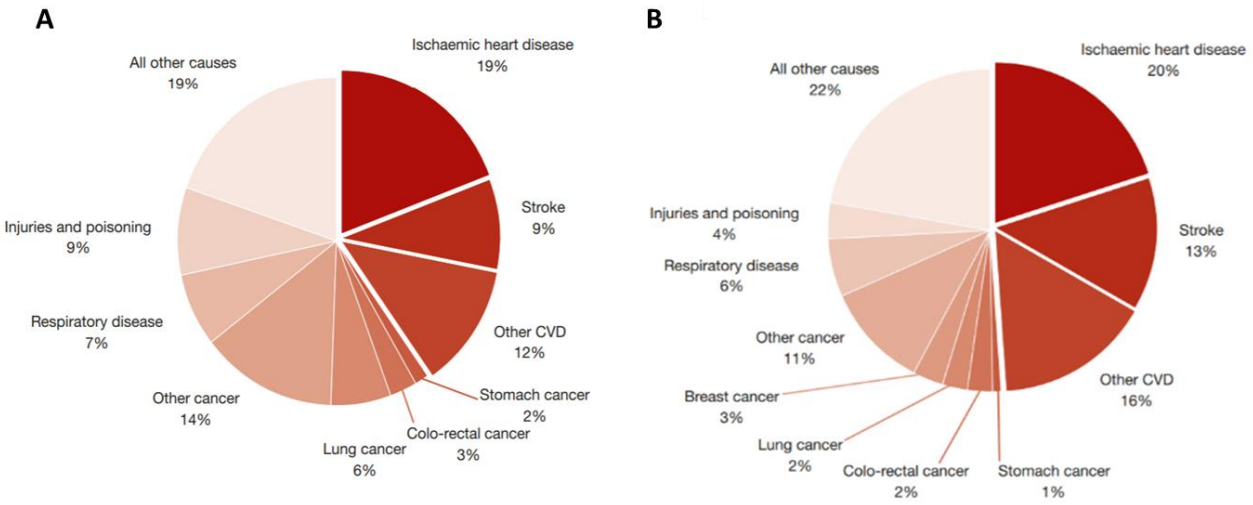

FIGURE 1| Causes of deaths in Europe in 2016 in males (A) and females (B). Source: World Health Organization Mortality Database.

In Iberian Peninsula the scenario is very similar. In both Spain and Portugal CVDs are the first cause of death, according to the last national statistical records (Figure 2). These graphics show that almost 30% of the annual deaths are due to CDVs in both countries.

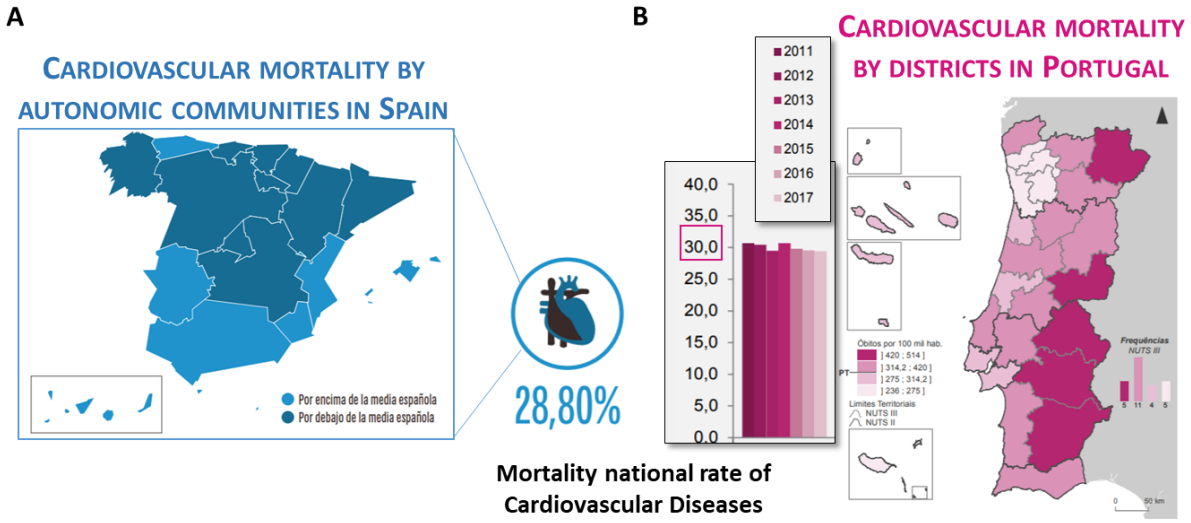

FIGURE 2| Mortality national rate by cardiovascular diseases in Spain in 2017 (A) and in Portugal between 2011 and 2017 (B) and its distribution through the country. Results are presented as a percentage of total number of deaths. Source: National institute of statistic of Spain and Portugal.

Despite of the numerous efforts taken so far to revert these numbers, unfortunately, they have not been reduced over the years. Therefore, the role of prevention become even more important, namely by controlling the possible risk factors associated with CVDs [3]. These risk factors can be classified as modifiable and non-modifiable. Non modifiable risk factors are those we cannot change. They include inheritance-positive family history, age and sex. CVDs are more frequent in men over age 45 and women over 55 years. The delay in the occurrence of CVDs in women, after menopause, is related to the protective effects exerted by female sex hormones on the cardiovascular system. On the other hand, modifiable risk factors are those we can influence by changing unhealthy lifestyle habits such as: smoking, harmful use of alcohol, insufficient physical activity, and high fat diets leading to obesity. In addition, some diseases such as elevated BP, hyperlipidaemia and diabetes are also important modifiable risk factors that can be controlled [2, 4].

ii. Hypertension as Risk Factor For Cardiovascular Diseases

Hypertension is defined as Systolic Blood Pressure (SBP) values ≥140 mmHg and/or diastolic BP (DBP) values ≥90 mmHg [5]. It can be classified into several degrees: High normal, Grade 1, Grade 2 and Grade 3 hypertension. The risk to develop a CVDs depends on the level of hypertension as well as the number of other CVDs risk factors. As so, risk can be stratified, simplifying the diagnosis and treatment decisions (Figure 3).

According to the World Health Organization (WHO), an estimated 1.13 billion people worldwide have hypertension, most (two-thirds) living in low- and middle-income countries. In Europe, the prevalence of hypertension in different countries seems to be about 30–45% of the overall population, with a steep increase with ageing [4].

During the last 2 decades, the number of diagnosed hypertensive patients has increased, as shown in the figure below regarding prescription of antihypertensive drugs (Figure 4). However, it is important to mention that a considerable percentage of hypertensive patients remain hidden, as shown by a recent study in Spain [6].

Other risk factors (RF), asymptomatic organ damage (OD) or disease	Blood Pressure (mmHg)			
	High normal SBP 130-139 or DBP 85-89	Grade 1 HT SBP 140-159 or DBP 90-99	Grade 2 HT SBP 160-179 or DBP 100-109	Grade 3 HT SBP ≥ 180 or DBP ≥ 110
No other RF		Low risk	Moderate risk	High risk
1-2 RF	Low risk	Moderate risk	Moderate to high risk	High risk
≥ 3 RF	Low to moderate risk	Moderate to high risk	High risk	High risk
OD, CKD stage 3 or diabetes	Moderate to high risk	High risk	High risk	High to very high risk
Symptomatic CVD, CKD stage ≥4 or diabetes with OD/ RFs	Very high risk	Very high risk	Very high risk	Very high risk

FIGURE 3| Stratification of total cardiovascular risk in categories of low, moderate, high and very high. RF: risk factors; OD: organ damage; SBP: systolic blood pressure; DBP: diastolic blood pressure; HT: hypertension; CVD: cardiovascular disease; CKD: chronic kidney disease. Source: Kjeldsen, 2018

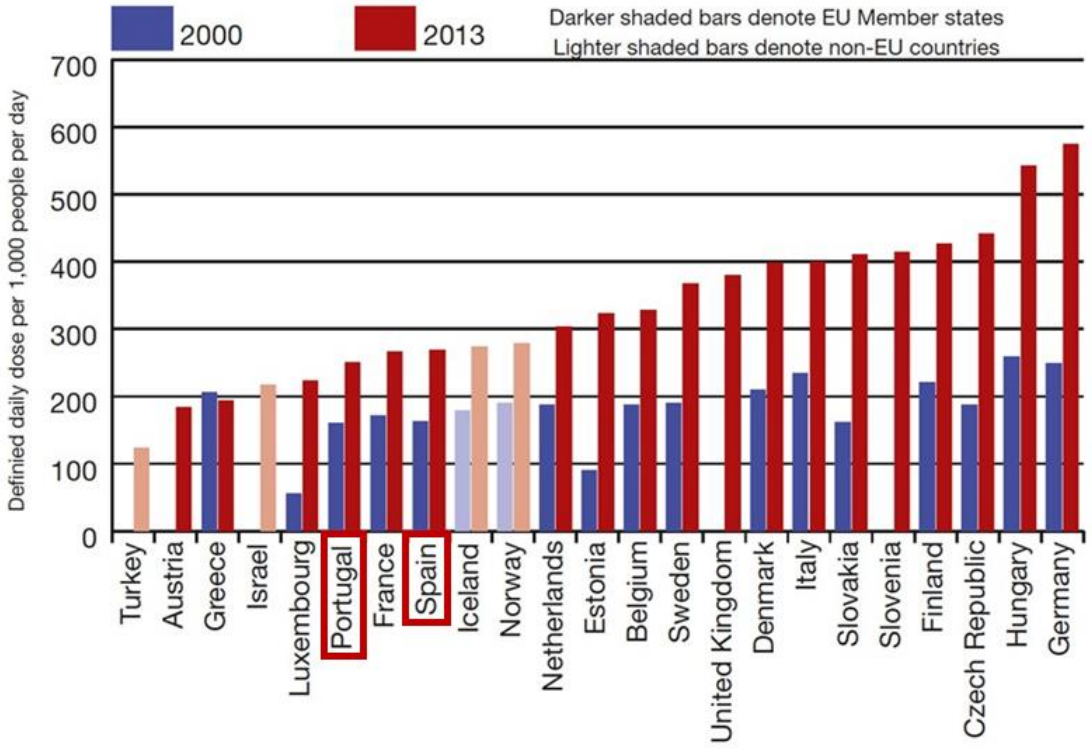

FIGURE 4| Antihypertensive drug prescriptions in 2000 (blue) and 2013 (red) in Europe. Source: European Heart Network in European Cardiovascular Disease Statistics 2017.

Hypertension may be classified as primary -of unknown origin- or secondary -with a known etiology, including renal, vascular, and endocrine causes. Primary, also known as essential hypertension, accounts for 90-95% of adult cases. As so, the majority of hypertensive patients exhibit an undefined etiology for a sustained increase in BP.

Since 2003, the Global Burden of Disease Study, organized by the WHO, has pointed hypertension as the most important global risk factor for morbidity and mortality. Indeed, hypertension is the strongest modifiable risk factor associated with the most important CVDs related to the generation of vascular damage in the heart, brain and kidneys; namely, coronary heart disease, heart failure, atrial fibrillation, left ventricular hypertrophy, cerebral stroke and renal failure [7].

Nowadays, more than 100 commercial drugs and drug combinations are available for treating hypertension. However, a substantial proportion of the hypertensive population has uncontrolled or suboptimally controlled hypertension [8], a fact that contributes to the growing global burden of CVDs [9].

iii. Model of Essential Hypertension

Due to the complexity of the pathophysiology of hypertension, experimental animal models are very useful for investigating specific factors that may be involved in the development or maintenance of the disease [10, 11].

The spontaneously hypertensive rat (SHR) strain was developed by Okamoto's group, by selecting and crossing rats of the Wistar Kyoto (WKY) strain that presented high BP [12]. The SHR rat is a good model for the study of human essential hypertension since it shares some physio-pathological similarities. Among them, a genetic predisposition to high BP without a specific etiology, an increase in peripheral total resistance and a similar response to drugs with BP lowering effects. Besides, there is evidence of alterations in vascular sympathetic neurotransmission, remodelling, oxidative stress and RAS, also present in human essential hypertension. Finally, there is a sexual dimorphism in the development of hypertension, with higher BP values in male rats. Based on these features the SHR was the chosen model of essential hypertension to perform experiments in this Thesis.

II. MECHANISMS IMPLICATED IN HYPERTENSION

The origin of essential hypertension is still unknown; however multiple mechanisms are recognized to be involved in the physiopathology of this disease. Among them, vascular remodelling, alterations in renin-angiotensin system (RAS), sympathetic nervous system (SNS), and oxidative stress are briefly described below, due to their importance in this work.

i. VASCULAR REMODELLING

Blood vessels are capable of structural changes in a dynamic process called vascular remodelling, which involves cell growth, death, phenotypic change and migration, as well as deposition of collagen, fibronectin, and other components of the extracellular matrix [13]. Vascular remodelling is also accompanied by altered vascular tone (decreased relaxation and/or increased contraction), associated with mild inflammation and fibrosis [14, 15]. Vascular remodelling can be a physiological process to adapt blood vessels to new hemodynamic or humoral conditions. However, in the end, it may be deleterious and contribute to CVDs development.

a. Vascular Wall

Blood vessels are an intrinsic part of cardiovascular system since they are the structures through which blood flows throughout the body. All blood vessels follow the same general histological structure. Nevertheless, they present differences that histologically differentiate arteries from veins (Figure 5).

The vascular wall is formed by 3 different tunicas or layers. The vascular layer that is in direct contact with the blood flow is the tunica intima (commonly called the intima layer), made up mainly of endothelial cells. The next layer is the tunica media (known as the media layer), with vascular smooth muscle cells (VSMC) and connective tissue. The outermost layer is known as the tunica adventitia (the adventitia layer), and it is composed mainly of fibroblasts and connective tissue, mainly collagen [16].

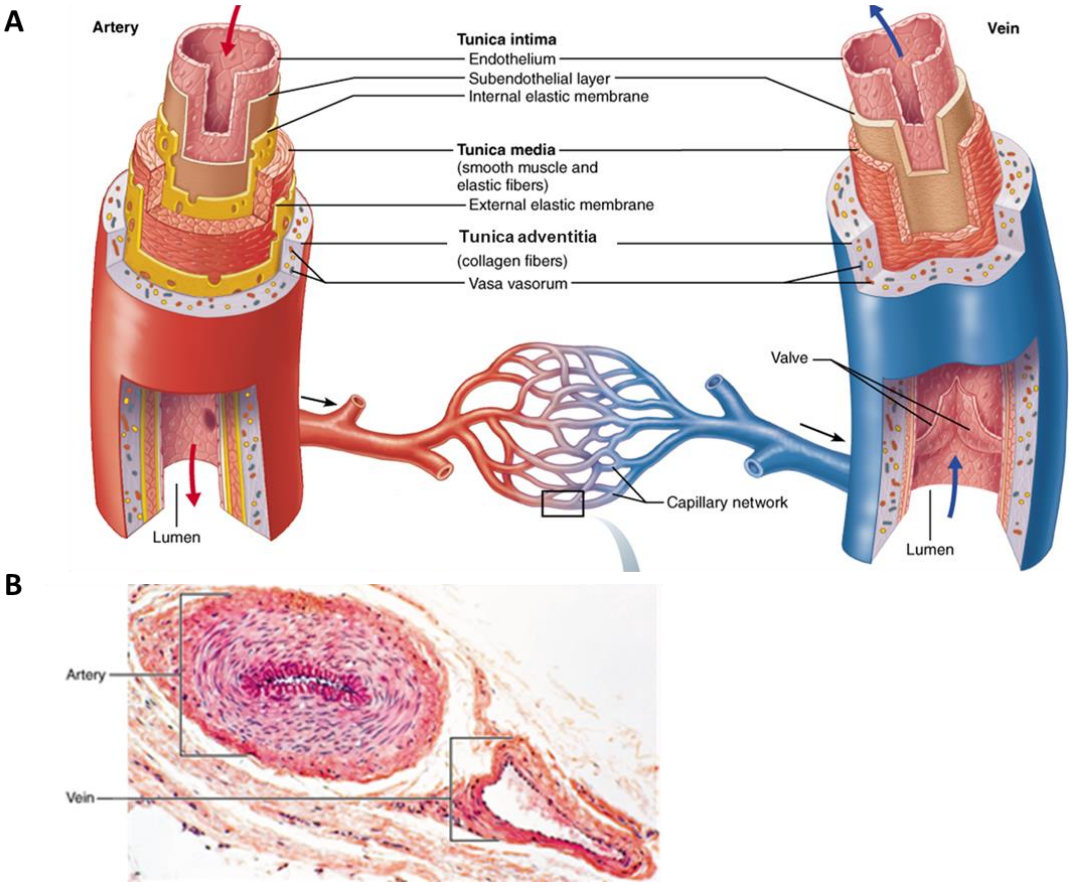

FIGURE 5| Schematic representation of the vascular wall (A) and histological representative images of an artery and a vein (B). All layers of the vascular wall are represented (adapted from Pearson Education Inc., 2014).

As the thickness of vein walls is typically much less than the diameter of the lumen (i.e., proportionately much thinner than arteries carrying a similar volume), veins tend to appear flattened or collapsed in cross-section of histological preparations (in contrast to arteries, which tend to appear more round) (Figure 5B).

The intima is the inner layer of a blood vessel, consisting in a very thin lining of simple squamous endothelial cells supported by a similarly thin layer of connective tissue. In arteries this layer is composed of an elastic membrane lining, and smooth endothelium that is covered by elastic tissue [17]. The endothelium has key physiological roles to regulate blood vessels function since endothelial cells are situated at a critical location, between the blood and all other body cells. Endothelial cells secrete substances which control local blood flow and blood coagulation. The integrity of the endothelium is

crucial, since damage can lead to atherosclerosis, abnormal clotting, and reduced vasodilatation.

The media is the middle layer of a blood vessel and, in most arteries, it is the thickest of the three tunics. The thickness of the media is generally proportional to the overall diameter of the vessel. The media of arteries is generally thicker than the media of veins of comparable diameter. The media consists of VSMC and elastic tissue in varying proportions. Elastic arteries have the highest proportion of elastic tissue while muscular arteries have the highest proportion of VSMC [17]. In most vessels, VSMC are arranged circumferentially, so the plane of section across an artery can be deduced by the orientation or shape of VSMC nuclei. The media elastic tissue is organized in concentric lamellas which alternate with VSMC forming the lamellar unit, which is the functional unit of the vessel wall. The number of lamellar units is directly proportional to the tension supported by the wall, and is maximal in the aorta.

The adventitia is the outer layer of the artery, composed of connective tissue with collagen and elastic fibres. The fibres of adventitial connective tissue tend to be more concentric around the vessel, and often somewhat denser than the surrounding connective tissue (fascia). In some blood vessels, the adventitia contains sympathetic innervation, and, in large arteries this layer harbours vasa vasorum, small vessels which supply oxygen and nutrients to the arterial wall. The adventitial layer is surrounded by adipose tissue that secrets adipokines, that also regulate the tone of the vessel [17].

b. Importance of the tunicas in vascular remodelling

VSMC are the most abundant cell type in the arterial wall and are essential in maintaining vessel structure and function, and so, playing a key role in arterial remodelling [18]. VSMC display a high degree of plasticity [19]. Under physiological conditions, VSMC have a contractile phenotype, which facilitates the contraction and dilation of the vasculature and, in turn, regulates the blood flow. Upon biological stress signals or vascular injury, VSMC respond by losing contractility markers and differentiate towards a synthetic phenotype that express proteins involved in proliferation and migration [20].

The adventitia has been mostly disregarded as participating in the development of vascular disease, whereas the media and intima have been seen as the predominate layers involved in the formation of atherosclerotic plaques and vascular remodelling [21]. Historically, it has been described as a purely structural asset to the arterial wall, presenting a support function for the underlying layers of the vessel, because the most common cell in the adventitia, the fibroblast, was seen mostly as a producer of the extracellular matrix (collagen types I and III) [22]. However, this has changed as multiple studies over the past decade have strongly refuted the idea that the adventitia plays only a passive role in the vasculature. Indeed, some studies demonstrate that adventitia is the most complex compartment of the vessel that integrates key regulators of vessel wall function [23, 24]. Moreover data demonstrate that wall changes in the adventitia occur early in the course of vascular disease development, before changes are seen in the intima or the media [21]. In the literature the influence of intima to media layers and vice versa is well established: endothelial cells can release mediators that alter the tonus of VSMC and VSMC can also influence the activity of endothelial cells [25]. Recent data has also highlight the interplay of intima with adventitia since endothelial cells have been shown to release mediators able to alter vascular sympathetic tonus [25-27].

c. Classification of arteries

Since arteries play a major role in nourishing organs with blood and nutrients, they are always under high pressure. To accommodate this stress, in the media layer of larger arteries, they present an abundance of elastic tissue and less smooth muscle. When an artery reaches a particular organ, it undergoes further division into smaller vessels, which have more smooth muscle and less elastic tissue. As the diameter of the blood vessels decreases, the velocity of blood flow also diminishes.

There are two main types of arteries found in the body: the elastic and the muscular arteries. Muscular arteries include the anatomically named arteries like the brachial artery, the radial artery, and the femoral artery for example. Muscular arteries contain more VSMC in the tunica media than the elastic arteries. Elastic arteries are larger arteries nearest the heart (aorta, carotid and pulmonary arteries) that contain much

more elastic tissue in the tunica media. This feature allows to maintain a relatively constant pressure gradient along the cardiac cycle.

Muscular arteries branch into small diameter arteries with fewer VSMC layers called resistance arteries. They contribute significantly to the creation of the resistance to flow and regulation of blood flow through the degree of the contraction of the VSMC and lumen narrowing. Resistance arteries divide into arterioles, which contain only 1 VSMC layer and these branch into meta-arterioles and capillaries, which have the role of exchange nutrients and gas [17].

d. Classification of vascular remodelling

As stated above, blood vessels undergo structural changes, which modify their geometry and composition. According to the alterations in lumen size, vascular remodelling can be classified as outward or inward remodelling, if lumen expands or narrows, respectively. According to changes in wall cross sectional area, it can be classified as hypertrophic (increased), hypotrophic (decrease) or eutrophic (no change) (Figure 6) [20].

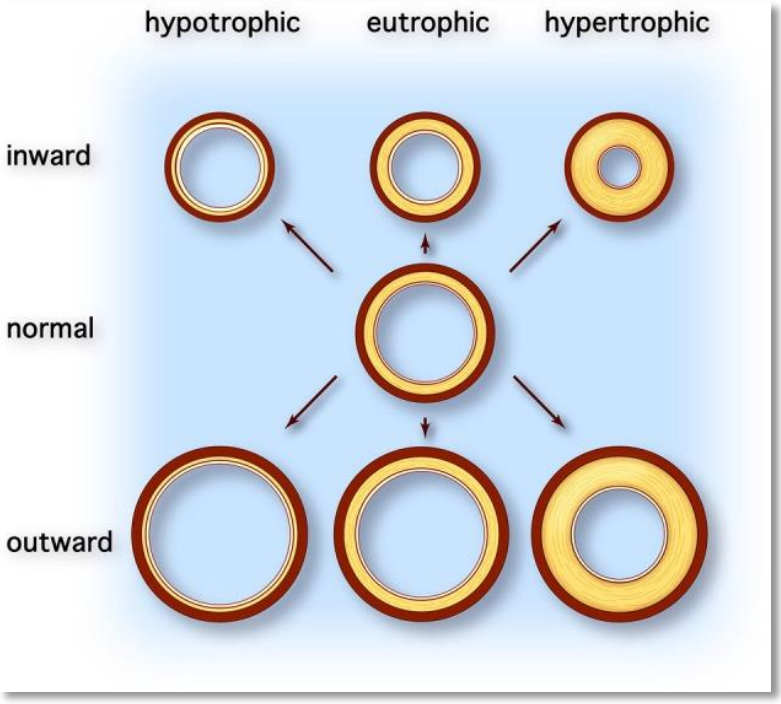

FIGURE 6| Types of vascular remodelling according to geometrical changes. Different types of arterial remodelling can be distinguished: hypotrophic (left column), eutrophic (center column) and hypertrophic (right column). In addition remodelling can be either inward or outward (from Mulvany *et al.,* 1996).

In addition, an important parameter is the wall thickness/lumen, since it has been demonstrated in resistance arteries from human subjects that an increase in wall/lumen ratio, is associated with higher risk of adverse cardiovascular events [28, 29].

Vascular remodelling characteristics are different depending on the underlying pathophysiology (e.g., aneurysms, essential or secondary hypertension, diabetes, etc.) and arterial site (e.g., central elastic arteries or peripheral resistance arteries) [14]. In resistance arteries, the main characteristic is a decrease in lumen diameter and an increase in media thickness, sometimes resulting in hypertrophic or eutrophic remodelling. This alteration leads to an increase in total peripheral vascular resistance [30]. Large arteries however do not have the ability to constrict in response to stress, and therefore show outward hypertrophic or eutrophic remodelling, characterized by increased vessel diameter usually accompanied by thickened intima and media layers of the vascular wall [14].

The thickening of the arterial wall may be caused by intima hyperplasia, media hypertrophy and hyperplasia of VSMC. Upon activation of VSMC by certain stimulus, they activate and change phenotype. This, in turn, alter their function and increase the deposition of extracellular matrix material. These changes in composition contribute to alterations in vascular function, both in active function of the endothelial and VSMC, and also in the mechanical behaviour of the arterial wall, particularly when collagen deposition is increased, leading to fibrosis [28, 31].

e. Mechanisms implicated in hypertensive vascular remodelling

Remodelling of large and small arteries contributes to the development and complications of hypertension (Figure 7) [15].

Vascular remodelling can be a consequence of alterations in hemodynamic parameters. For example, sustained high BP can be a stimulus for wall growth or lumen reduction, since it would increase wall stress (wall stress=pressure x vessel diameter/2xwall thickness). This parameter has to remain relatively constant and, if increased, it may cause vessels rupture. Therefore, if pressure is increased for a long time, this stimulates either wall growth or lumen size reduction. These geometrical changes would restore

wall stress to initial conditions [32]. Despite the fact that this is a physiological adaptive response, if a stimulus is chronically maintained, it will be deleterious for arterial function.

In addition to alterations in hemodynamic conditions, several humoral factors can be implicated in vascular remodelling, such as reactive oxygen species (ROS), angiotensin II (Ang II), noradrenaline (NA), and several growth factors.

ROS are well known to be involved in the pathogenesis of hypertension. Among other actions, they are implicated in vascular cell growth and death; specifically, superoxide anion ($\bullet O_2^-$) induces proliferation and, hydrogen peroxide (H_2O_2), may induce apoptosis via a protein kinase C–dependent mechanism [33].

Ang II also modulates apoptosis throughout the activation of angiotensin receptor type 1 (AT_1) and type 2 (AT_2) [34, 35]. Ang II also mediates inflammation in the vasculature mainly by stimulation of $\bullet O_2^-$ production and subsequent activation of redox-dependent signalling cascades [15, 36]. Further proof of the role of AT_1 receptors in vascular remodelling is found in studies which demonstrate that AT_1 receptor antagonists regress vascular structural changes in hypertensive patients [36].

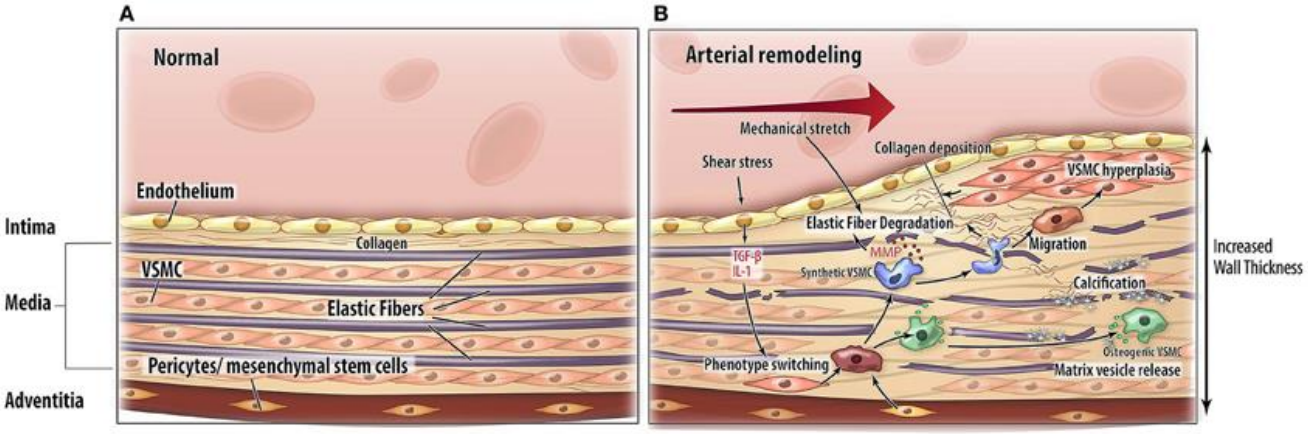

FIGURE 7| Pathophysiological mechanisms of arterial remodelling. Cross sectional schematic view of the arterial wall. (A) Normal situation. (B) Arterial remodelling. Arterial remodelling is characterized by thickening of the wall. Elastic fiber degradation, extracellular matrix calcification and collagen deposition lead to adaptation of the vascular wall. Abbreviations: TGF-β, transforming growth factor-beta; IL-1, interleukin 1; MMP, matrix metalloproteinases; VSMC, vascular smooth muscle cell (from J. van Varik *et al.*, 2012).

ii. ENDOTHELIAL DYSFUNCTION

Accumulating evidence has demonstrated that endothelial functions are essential to ensure proper maintenance of vascular homeostasis [37]. Endothelial cells are exposed to the shear stress resulting from blood flow and can convert mechanical stimuli into intracellular or biochemical signals (proliferation, apoptosis, migration, remodelling, etc.). In fact, the endothelium can modulate the vascular tone by synthesizing and releasing a variety of factors, including vasodilators, like prostaglandins, nitric oxide (NO), and endothelium-dependent hyperpolarization factors, as well as vasoconstrictors, such as endothelin-1, Ang II, thromboxane A2, and ROS. All these molecules influence vascular tone [14].

A healthy endothelium presents an athero-protective capacity, through the promotion of vasodilation, its antioxidant and anti-inflammatory effects, as well as the inhibition of leukocyte adhesion and migration and VSMC proliferation and migration. Also, a healthy endothelium has anticoagulant and pro-fibrinolytic effects, as well as an inhibitory action on platelet aggregation [38]. All these aspects occur due to a balance between endothelium-derived relaxing and contracting factors (Figure 8). Disturbance of this balance causes endothelial dysfunction and it is a hallmark of an extensive range of CVDs associated with pathological conditions toward vasoconstriction, thrombosis, and inflammatory state.

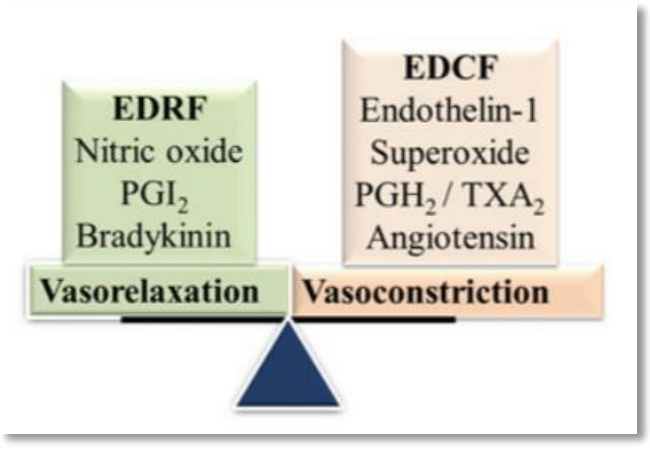

FIGURE 8| The balance between endothelium-derived relaxing factors (EDRF) and endothelium-derived contracting factors (EDCF). PGI_2, prostacyclin; PGH_2, Prostaglandin H2; TXA2, Thromboxane A2 (from Konukoglu *et al*., 2017).

Endothelial dysfunction has a critical role in the development of hypertension and contributes to the progression of organ damage [38, 39].

In hypertension, sustained elevation of systemic pressure in the microvasculature leads to premature aging and increased turnover of endothelial cells. As a result, there is a disruption of multiple endothelial-dependent mechanisms that can contribute to maintain hypertension (Figure 9). Endothelial dysfunction is associated with a reduction of the NO bioavailability, either due to decreased production by NO synthases (NOS), or to increased destruction by ROS.

Endothelial dysfunction in hypertension is also linked to impairment in the response of VSMC to vasodilators, elevated sensitivity of endothelial cells against vasoconstrictors, increased production of vasoconstrictor substances, or elevated shear stress. All these leads to functional changes in the vasculature with a predominant and deleterious constrictive tone [38].

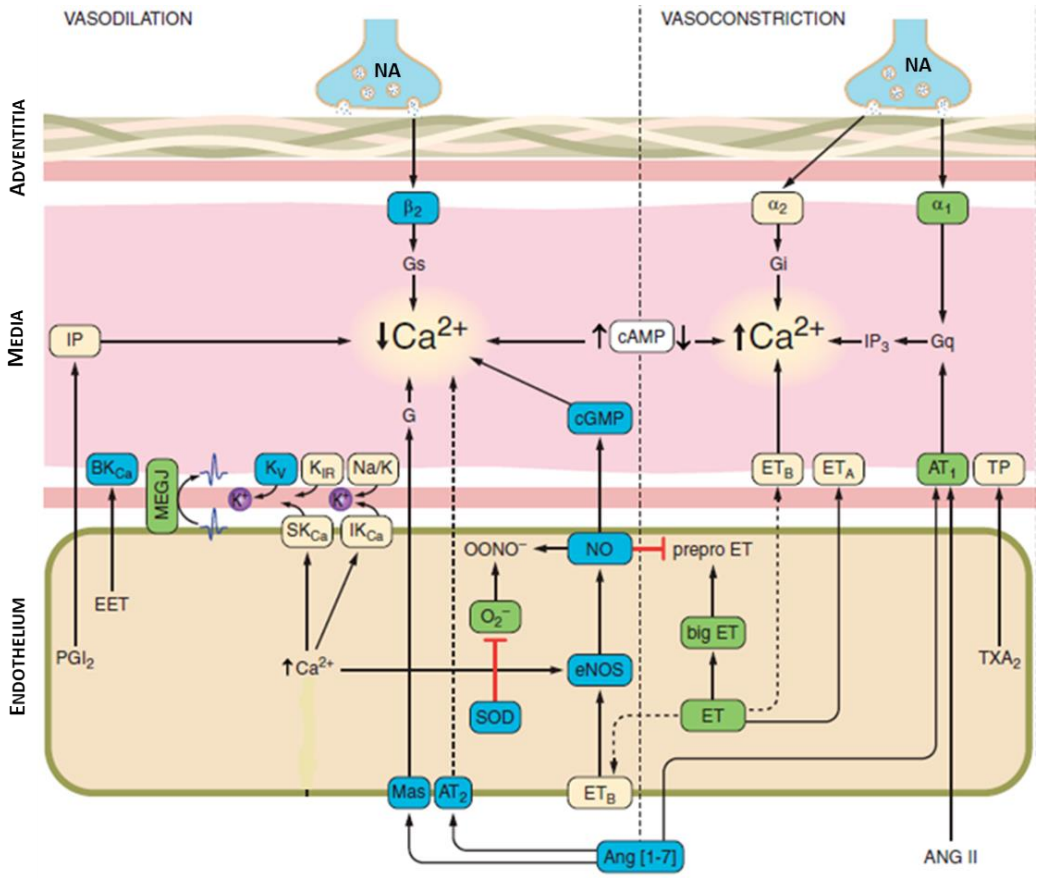

FIGURE 9| Representative scheme of endothelial control of vascular tone. Vasodilator pathways include the prostacyclin, endothelium-derived hyperpolarization, angiotensin, and nitric oxide (NO) pathways that are additionally influenced by endothelin and superoxide production. Vasoconstrictor pathways include endothelin, adrenergic, angiotensin, and thromboxane pathways. Endothelial and smooth muscle membrane hyperpolarization has been depicted by an action potential symbol. α_1, alpha 1-adrenergic receptor; α_2, alpha 2-adrenergic receptor; ANG II, angiotensin II; AT_1, angiotensin 1 receptor; AT_2, angiotensin 2 receptor; β_2, beta 2-adrenergic receptor; big ET, big endothelin; BKCa, large conductance calcium-activated potassium channel; Ca^{2+}, molecular calcium; EET, epoxyeicosatrienoic acid; eNOS, endothelial nitric oxide synthase; ET, endothelin; ET_A, endothelin A receptor; ET_B, endothelin B receptor; Gi, inhibitory G protein; Gq, phospholipase C activating G protein; Gs, stimulatory G protein; IKCa, intermediate conductance calcium activated potassium channel; IP, prostacyclin receptor; IP_3, inositol triphosphate; K^+, molecular potassium; KIR, inward-rectifying potassium channel; Kv, voltage-gated potassium channel; Mas, angiotensin mas receptor; MEGJ, myoendothelial gap junction; Na/K, sodium potassium ATPase exchanger; NA, noradrenaline; $O_2^{\cdot-}$, superoxide; $OONO^-$, peroxynitrite; PGI_2, prostacyclin (also known as prostaglandin I2); prepro ET, prepro endothelin; SKCa, small-conductance calcium-activated potassium channel; SOD, superoxide dismutase; TP, thromboxane receptor; TXA2, thromboxane A2 (adapted from Morton *et al*., 2016).

iii. Renin-Angiotensin System

a. The RAS and vascular function

The RAS is a very complex system with many peptides and receptors involved. Briefly, angiotensinogen is released from the liver and is cleaved in the circulation by the enzyme renin, secreted from the juxtaglomerular apparatus of the kidney, to form angiotensin I (Ang I). Then, Ang I is transformed to Ang II by angiotensin converting enzyme (ACE), which is expressed in high levels on the surface of endothelial cells in the pulmonary circulation [40, 41]. Ang II, considered as the most powerful active peptide of the RAS, acts on specific receptors: AT_1 and AT_2, being the AT_1 receptor predominant.

Recently, new peptides, enzymes and receptors with biological activity have been reported as components of the RAS [40-42]. Among them, the peptide angiotensin-(1-7) (Ang 1-7), that is mainly formed directly from Ang II, by angiotensin converting enzyme 2 (ACE2), and the peptide alamandine. These peptides bind, respectively, to receptor Mas and Mas-related G protein coupled receptor D (MrgD), and counteract the main effects of Ang II through AT_1 receptor (Figure 10).

With these new findings the RAS is now classified in 2 axis, the classical and the non-classical. The classical axis includes the renin/ACE/Ang II/AT_1 receptor. The non-classical axis includes the ACE2/Ang 1-7 and alamandine/acting on Mas and MrgD receptors [40]. These 2 axis seem to have opposing actions on the cardiovascular system. The classical RAS axis releases Ang II, which promotes vasoconstriction and increase oxidative stress, fibrosis, and inflammation by activating AT_1 receptor. The non-classical RAS axis leads to Ang 1-7 and alamandine acting on Mas and MrgD receptors, which cause an increase of NO bioavailability and a decrease of oxidative stress, through inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [40]. Also these new RAS players seem to be protective avoiding remodelling and vascular wall structural alterations [41]. Therefore, dysregulation of the classical and non-classical RAS axis -either in the enzymes and subsequent peptide production or in the expression of the specific receptors- may be implicated in pathological situations such as hypertension.

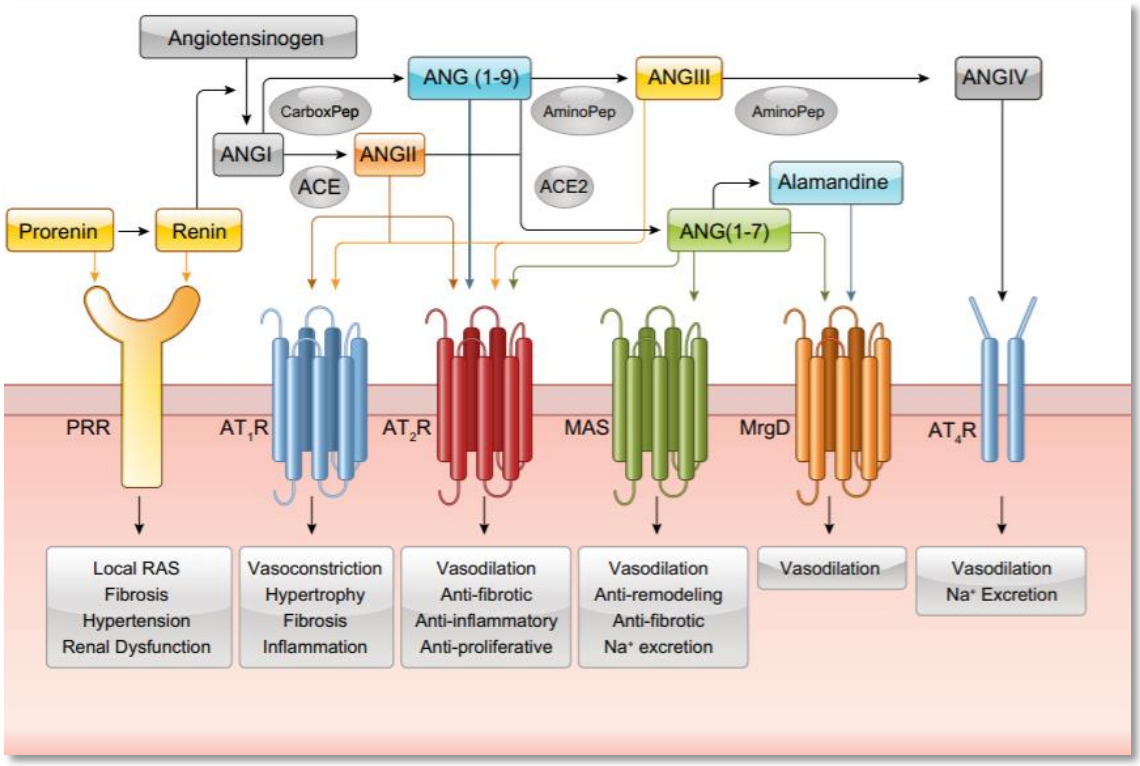

FIGURE 10| Identified angiotensin peptides and receptors in RAS signaling. ANG, angiotensin; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; CarboxPep, carboxipeptidase; AminoPep, aminopeptidase; PRR, (pro)renin receptor; AT_1R, angiotensin type-1 receptor; AT_2R, angiotensin type 2 receptor; AT_4R, angiotensin type 4 receptor; MAS, Ang-(1-7) receptor Mas; MrgD, MAS related G-protein coupled receptor D (from Forrester *et al*., 2018).

Experimental evidence also support the concept that local RAS is present in different organs including the heart and kidney, and in VSMC, contributing to local regulation of tissue and organ function [43]. Indeed, the synthesis of several components of the RAS in the heart and vessels, or their uptake from plasma, makes possible the synthesis of Ang II locally. Evidence demonstrate that angiotensinogen, ACE, Ang I, and Ang II are synthesized in the VSMC and the presence of renin has been found in these cells [43]. The origin of vascular renin, *in vivo*, might be related to local production or be related to its uptake from the circulation, which is known to play an important role in the etiology of vascular complications during hypertension [43].

b. RAS alterations in hypertension

The RAS influences a large range of homeostatic and modulatory processes including regulation of salt and water balance, vasoconstriction, cell/tissue remodelling. Therefore, an alteration in RAS is linked to alterations in the cardiovascular system.

In fact, the involvement of the RAS in the pathophysiology of hypertension has been known for many years and various components of the systemic RAS are pharmacological targets to treat CVDs, including renin inhibitors, ACE inhibitors, and AT_1 receptor antagonists [44, 45]. All of these drugs are primarily designed to block and/or reduce the harmful effects of Ang II. There is, however, increasing evidence that in addition to Ang II, many other angiotensin peptides such as Ang III (Ang 2-8), Ang IV (Ang 3-8), Ang 1-9 and Ang 3-7 have important physiologic effects. These new findings have opened new advances for the development of novel drugs targeting the RAS to better treat hypertension. New strategies include gene therapy to suppress angiotensinogen at the RNA level, recombinant ACE2 protein, and novel bispecific designer peptides [46].

Below, there is information on the effects of the different RAS receptors, cantered on the role on vascular physiological and pathophysiological processes.

c. RAS receptors

AT_1 Receptor

The AT_1 receptor is predominantly expressed in various tissues throughout the cardiovascular system including VSMC, endothelium, heart and kidney [47].

The effects of AT_1 receptor activation by Ang II varies depending on the tissue. Among other actions, Ang II binding to AT_1 receptor leads to VSMC vasoconstriction and hypertrophy, cardiac myocyte hyperplasia, aldosterone release, renal sodium reabsorption and sympathetic nervous adrenergic facilitation in the SNS [48]. AT_1 receptor triggers several intracellular signalling pathways through the activation of various protein kinases (e.g. activation of phospholipase C, generation of inositol phosphate and Ca^{2+} release, mitogen-activated protein (MAP) kinase activation, etc.). This signalling pathway is associated with increased BP, inhibition of NO production,

vascular remodelling, promotion of tissue inflammation and fibrosis, increased oxidative stress and increased aldosterone production [47]. AT_1 receptors in the VSMC once activated increase the intracellular pool of Ca^{2+}, which in turn, lead to VSMC contraction. This vasoconstriction, ultimately, lead to an increase in BP [49].

AT_1 receptor antagonists are an important pharmacological tool consistently used for the treatment of hypertension and diabetic nephropathy.

AT_2 Receptor

AT_2 receptor expression is very high in embryonic tissues and decreases shortly after birth in most organs. Nevertheless, lower AT_2 receptor expression is maintained in kidneys, heart, blood vessels and brain [50, 51]. AT_2 receptor has been shown to be constitutively expressed at a low level in the physiological state, but is markedly up-regulated in certain cardiovascular disorders, including hypertension [52].

Although the physiological/pathophysiological roles of the AT_2 receptor have not been fully clarified, there is an increasing interest in AT_2 receptor functions in the cardiovascular system [53]. Current knowledge suggests that under normal physiologic conditions, AT_2 receptor counterbalances the effects of AT_1 receptor, inhibiting cell proliferation and differentiation, promoting vasodilation, and reducing inflammation and oxidative stress [51]. It is known that AT_2 receptor stimulation causes vasodilation through endothelial nitric oxide synthase (eNOS) activation, among other molecules [53, 54], and pharmacological stimulation of AT_2 receptor promotes natriuresis and reduces BP [55]. Up-regulation in AT_2 receptor expression has support their implication in wound healing, tissue remodelling and reduced inflammation [52].

All of these beneficial effects have suggested the possibility that AT_2 receptor agonist therapy may be used in CVDs in the near future [56].

Mas Receptor

The Mas receptor is a proto-oncogene that is expressed in brain, kidneys, heart and blood vessels of both humans and mice [57].

Gene and pharmacological manipulation studies reveal that Mas receptor is involved in natriuretic, vasodilatory, antiproliferative and anti-inflammatory responses, and in BP lowering in various animal models of hypertension, such as salt-sensitive hypertension [58]. The most reported signalling pathways linked to Mas stimulation in the vasculature [59] and heart [60] are those related to prostaglandins, and NO production through activation of eNOS, highlighting the importance of the Ang 1-7/Mas axis as a putative regulator of endothelial function.

MrgD Receptor

Mas-related genes (Mrgs), a large family of G-protein coupled receptors (GPCRs), have been divided into subfamilies established by sequence homology (MrgA, MrgB, and MrgC), and some more single genes (MrgD, MrgE, MrgF, MrgG, MrgH, and Mas 1) [61].

MrgD is widely expressed in sensory neurons in the dorsal root spinal ganglia, but it can be located in other organs in lower levels, namely in arteries and in the heart [41].

The MrgD receptor ligand (alamandine) only differs from Ang 1-7 in one amino acid (an alanine instead of aspartate in the N-terminal domain). Therefore, the biological actions of these peptides are very similar. Alamandine and Ang 1-7 have been demonstrated to produce endothelial-dependent vasodilation, and were able to modulate the baroreflex sensibility facilitating bradycardia [42]. However, some different actions between these two peptides have also been reported. For example, while Ang 1-7 exhibits antiproliferative effects on tumour cells, this effect was not observed for alamandine [41, 62].

In SHR, alamandine induced vasodilatation and demonstrate antihypertensive effects [62]. Besides, in isoproterenol-treated rats, almandine had pronounced cardioprotective effects [62]. These findings, and the fact that alamandine can be easily administered

orally, opens new perspectives for exploring the therapeutic potential of Ang 1–7–related peptides in the treatment of CVDs [62].

iv. Sympathetic Nervous System

The autonomic nervous system is a major regulator of the cardiovascular system. The SNS is the component of the autonomic nervous system that is responsible for controlling the human body's reaction to situations of stress or emergency, while the parasympathetic nervous system is generally responsible for basal organ and system function.

These two divisions regulate heart rate (HR) and BP in the short-term. Parasympathetic modulation decreases the HR and cardiac contractility, whereas activity of the sympathetic branch opposes these effects, and also regulates peripheral vasoconstriction. In fact, most blood vessels lack parasympathetic innervations and their diameter is regulated by SNS input, so that they have a constant state of sympathetic tone.

The SNS has an integral role in the regulation of HR and contractility, vascular tone and fluid volume. The activation of SNS in the heart releases NA, a neurotransmitter that binds to adrenoceptors (primary β-adrenoceptors), increasing HR, contractility and velocity of electrical impulse conduction, resulting in an increased cardiac output and arterial BP. On the other hand, NA can also bind to α-adrenoceptors in the VSMC causing vasoconstriction, which also contributes to BP increase. α-adrenoceptors (α_1 and α_2) are the main type of receptors in blood vessels, but β-adrenoceptors (few β_1, β_2 and also β_3) are also present in the vasculature [63, 64].

a. Vascular sympathetic neurotransmission

In sympathetic nervous system, large axons, even those unmyelinated, are surrounded by a lipid-rich sheath of Schwann cells. The plasma membrane of these glial cells folds once around each fibre overlapping itself along the edges. In most cases, nerve fibres

travel in individual channels in the Schwann cells, but when nerve fibres are small they can bundled together within a single channel [65].

In SNS, the majority of the postganglionic endings release NA. NA is synthesized inside the nerve axon, stored within vesicles, and then released by the nerve when an action potential travels down the sympathetic nerve. Briefly, the amino acid tyrosine, transported into the sympathetic nerve axon, is converted to DOPA by tyrosine hydroxylase (TH), a rate-limiting step for NA synthesis. DOPA is then converted to dopamine by DOPA decarboxylase. Dopamine is transported into vesicles then converted to NA by dopamine β-hydroxylase. When an action potential traveling down the axon depolarizes the membrane, it causes Ca^{2+} to enter the axon. Increased intracellular Ca^{2+} causes the vesicles to migrate to the axonal membrane and fuse with the membrane, which allows the NA to diffuse out of the vesicle. The NA binds to the postsynaptic receptor and stimulates the effector organ response. Once in the synaptic cleft, most of the NA (~90%) is transported back into the nerve terminal by a neuronal reuptake transport system. NA can also diffuses into capillaries and is carried out of the tissue by the circulation. Therefore, high levels of sympathetic activation in the body increase the plasma concentration of NA and its metabolites. However, some of the NA released is metabolized within the extracellular space before reaching the capillaries. Additionally, a small amount of NA (~5%) is taken up by the postsynaptic tissue and metabolized by catechol-O-methytransferase and monoamine oxidase (Figure 11) [66].

Sympathetic vascular innervation varies widely among vascular beds. Generally, the density of sympathetic nerves increases as the arterial calibre decreases, so that small arteries have the densest innervation and, in these beds, sympathetic stimulation produces profound vasoconstriction.

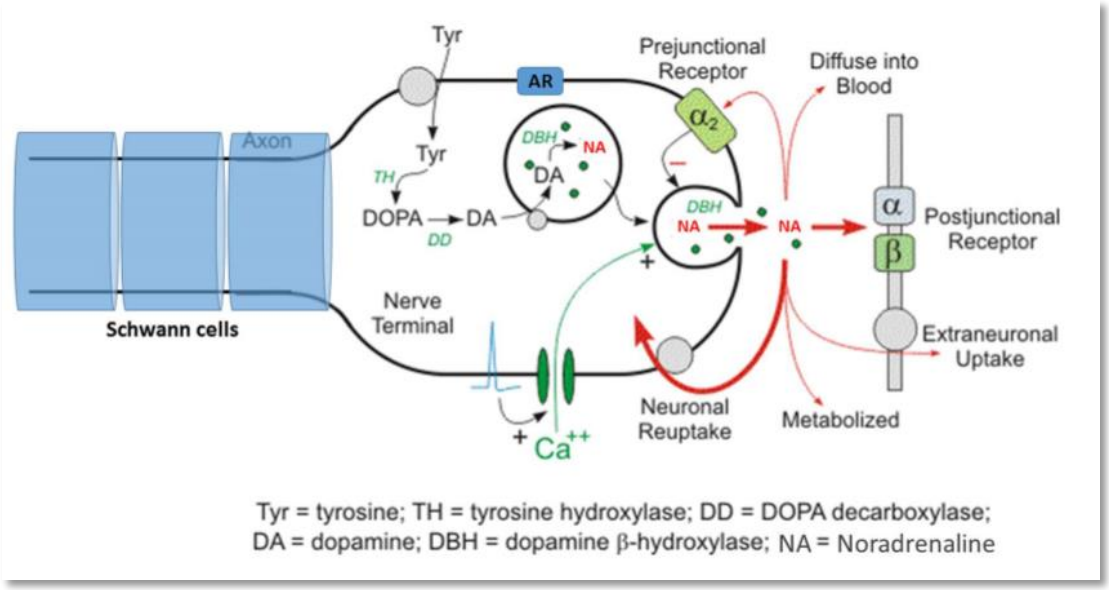

FIGURE 11| NA synthesis, storage, release and action. (Adapted from Cardiovascular Pharmacology Concepts, Richard E. Klabunde, Lippincott Williams & Wilkins, Second Edition)

b. Alterations of the SNS in hypertension

In hypertension, autonomic cardiovascular control is compromised, leading to a reduced parasympathetic tone and to an increased sympathetic influence to the heart and peripheral vessels [67]. Data from humans and experimental animal models of hypertension support the neurogenic hypothesis of hypertension, which considers sympathetic hyperactivation as the primary factor underlying the elevation of BP [68-71]. Several studies have corroborate this by different means: 1) measuring circulating blood levels of the adrenergic neurotransmitters (adrenaline and NA), 2) quantifying efferent postganglionic muscle sympathetic nerve activity (SNA) in peripheral nerves or 3) assessing regional NA and reuptake by adrenergic nerves with radiolabelled NA [67, 72]. Although the main causes of sympathetic hyperactivation remain undefined, alterations of reflexes and/or metabolic factors have been proposed as candidates [71]. Nevertheless, it is known that excessive activation of SNS has numerous deleterious effects such as increased peripheral resistance, left ventricular hypertrophy, or alteration in renal function with retention of salt and water, thus, contributing to the development and/or maintenance of hypertension [67, 73-76]. Moreover, in vessels, an increased SNA is responsible for vascular remodelling related to VSMC hypertrophy and fibrosis (Figure 12) [77].

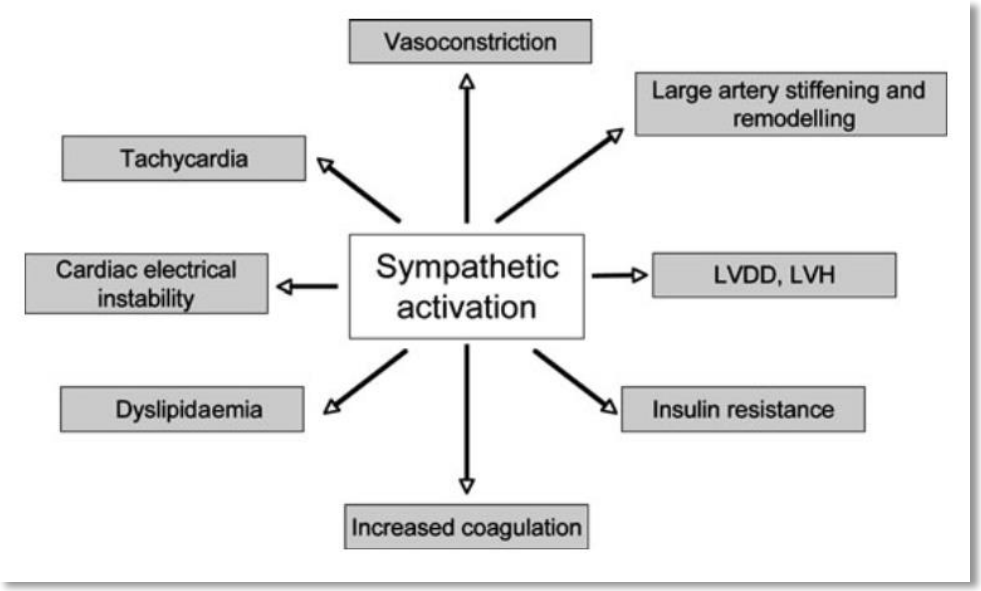

FIGURE 12| Schematic representation of the adverse cardiovascular and metabolic effects of sympathetic activation in hypertension. LVDD, left ventricular diastolic dysfunction; LVH, left ventricular hypertrophy (from G. Grassi et al., 2010).

c. Role of Ang II in SNS

It has long been proposed the existence of a bidirectional stimulatory interactions between Ang II and the SNS [78-80]. On one hand, renal sympathetic innervation is a major determinant of β_1-adrenergic receptor-mediated renin release, thus contributing to the control of circulating Ang II levels. Consistent with this, renal denervation suppress efferent sympathetic outflow reducing circulating renin, Ang II and aldosterone in experimental animal models and in patients with resistant hypertension [79]. On the other hand, AT_1 receptors are abundant at each synaptic relay of the autonomic nervous system to influence sympathetic and parasympathetic neurotransmission. Ang II enhances SNS neurotransmission by stimulating presynaptic release of NA from sympathetic nerves, facilitating sympathetic ganglionic transmission, inhibiting NA reuptake in nerve terminals, increasing the density of sympathetic innervation in cardiovascular organs, and enhancing vasoconstrictor responses to NA [80]. Emerging evidence suggests that activation of RAS (either through increased Ang II production, or enhanced AT_1 receptor expression and signalling) contributes to sympathetic hyperactivity and hypertension [81].

v. OXIDATIVE STRESS

Oxidative stress refers to the imbalance between ROS synthesis and elimination by antioxidant systems, which leads to excessive oxidative processes in cells and tissues. This alteration can be either related to an excess in ROS production by specific enzymes, or a decrease in buffering capacity non-enzymatic antioxidants or elimination of ROS by specific enzymatic systems [82].

Oxidative damage can induce alterations in the cardiovascular system and has been implicated in hypertension and other CVDs. Several ROS have been implicated in hypertension. Among them, one of the most important is $\bullet O_2^-$, considered a primary ROS, from which other ROS can be generated. One of the main deleterious actions of $\bullet O_2^-$ in the vascular system is the reaction with NO, which generates the production of peroxynitrite ($ONOO^-$). This is a much more stable ROS, which, in turn, promotes alterations of transcription factors, kinases, protein synthesis, and redox-sensitive genes. These changes induce functional and structural alterations in the vascular wall [83], including excessive contractility, alterations in VSMC growth/death, monocyte migration, lipid peroxidation and inflammation. All these alterations are known to be deeply involved in the pathogenesis and progression of vascular damage in hypertension and other CVDs [83, 84]. In hypertension, major source of $\bullet O_2^-$ are xanthine oxidase, uncoupled eNOS, NADPH oxidase, cyclooxygenase (COX) and the mitochondrial respiration chain (Figure 13) [85, 86].

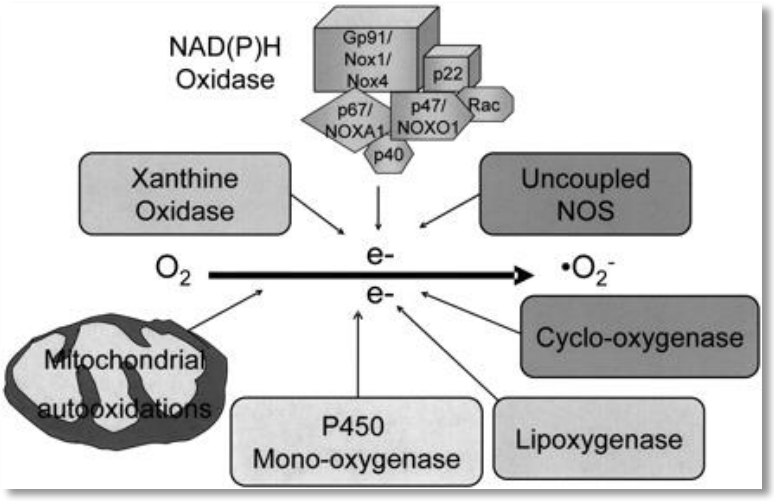

FIGURE 13| Enzymatic sources of superoxide anion ($\cdot O_2^-$). The major enzymes responsible for ROS generation in the vasculature include NAD(P)H oxidase, xanthine oxidase, and uncoupled NOS (from Paravicini *et al*., 2008).

a. NADPH oxidases

NADPH oxidases are a family of transmembrane enzymes capable of generating $\bullet O_2^-$ and it is the main enzyme system responsible for its production both in the vascular wall [87] and in the heart [88]. There are different isoforms of NADPH oxidase: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2, being Nox2 the most abundant in the cardiovascular system. This isoform consists of 5 subunits: a stabilizing subunit (p22phox), several regulator subunits (p40phox, p47phox, p67phox) and the catalytic subunit (gp91phox), which transfers electrons.

Activation of NADPH oxidase is regulated by many vasoactive hormones, growth factors (platelet-derived growth factor, transforming growth factors) and mechanical stimuli (shear stress and stretch) [85].

b. Role of Ang II on vascular ROS generation

Although NADPH oxidases are constitutive enzymes, Ang II represents one of the major vasoactive peptides involved in the regulation and activation of NADPH oxidase. Indeed, observations in experimental animals demonstrate that Ang II increases $\bullet O_2^-$ production by activation of NADPH oxidase in various vascular cell types, including VSMC, endothelial cells and fibroblasts [89, 90]. Vascular ROS production induced by Ang II takes place via AT_1 receptors, which stimulates both the expression of NADPH oxidase subunits and the activation of the enzyme [36]. On the other hand, ROS may regulate AT_1 receptor gene expression, which in turn modulates ROS production, thus creating a vicious circle [91].

III. FETAL PROGRAMMING OF HYPERTENSION

i. HYPOTHESIS

Along with genetic and lifestyle factors (sedentary, unhealthy diet, tobacco), it is now established that some stress factors during fetal life contribute to the development, in adult age, of CVDs [92-94]. The fetus can adapt to adverse intrauterine conditions, as malnutrition, oxygen deprivation, placental insufficiency, exposure to excess glucocorticoids (GC) or toxic substances, to ensure survival [95]. However, these increased susceptibility to develop CVDs, a phenomenon named Developmental Origins of Health and Disease or fetal programming [96].

Dr. David Barker was the first to advocate the hypothesis, by demonstrating that adaptive responses of the fetus to maternal undernutrition leading to low birth weight (LBW), are associated with the development of coronary heart disease and hypertension in adult life [92, 97, 98]. Over the past years, several epidemiological studies in populations exposed to starvation and intrauterine stress have confirmed this association and validated Dr. Barker´s hypothesis. Thus, the association between LBW and increased cardiovascular risk, namely of increased BP is now well established. This process is known as fetal programming of hypertension (FPH) [99-102].

ii. DETERMINANTS

The inverse relationship observed between LBW and hypertension may be due to fetal alterations induced by exposure to a suboptimal environment during gestation [97]. During intrauterine period, tissues and organs go through critical stages of development and the fetus has high plasticity. This plasticity represents also a high sensibility to any alteration in the environmental factors around the fetus. Therefore, its development is not only determined by the fetal genome, but also by maternal health and by the placental capacity to supply nutrients and oxygen [103-105]. When the fetus is exposed to a suboptimal environment which compromises growth at this critical period of development, the fetus can adapt to ensure survival. However, in long-term, this early

adaptive changes may lead to alterations in organ structure and function, with long term consequences. In particular, fetal exposure to stress conditions, has been demonstrated to lead to permanent alterations in the structure and function of key organs implicated in cardiovascular control resulting in increased risk for hypertension and CVDs development [95, 106]. Furthermore, the timing of the exposure to the adverse factor along gestation, mediates different fetal growth trajectories and the severity or nature of the long term cardiovascular alteration [107].

Several adverse factors during intrauterine life have been demonstrated to contribute to inadequate fetal development and fetal programming. The most important are malnutrition [107, 108], oxygen deprivation [109-111], placental insufficiency [112], exposure to excess GC [113-115], toxic substances (alcohol, tobacco) [116-118] and environmental pollutants [119].

a. Suboptimal fetal nutrition

An inadequate nutrition of the fetus may occur due to maternal malnutrition or to inadequate placentation; both have been demonstrated to be well-known fetal insults responsible for FPH.

Maternal malnutrition was proved to contribute to FPH in studies carried out in the offspring of women that suffered from starvation during pregnancy in periods of war [100, 120]. Later epidemiological studies carried out in different countries and in populations of different ethnic origin, in both men and women, have provided additional evidence that programming is a consequence of malnutrition, and is not the result of confounding variables [121-124].

On the other hand, placenta is fundamental for fetus´ nutrition as it controls oxygen and nutrients supply, and also is the source of specific hormones involved in fetal growth and development [125]. To achieve these functions, placenta performs a remarkably diverse range of activities, including active and passive transport, endocrine secretion, immunological protection, and xenobiotic detoxification. As well as being multifunctional, the placenta is also an organ with high plasticity, capable of considerable structural and functional adaptations that help to mitigate adverse

maternal insults [126]. An alteration in placenta´s function, as in of preeclampsia or placental insufficiency, may result in fetal development changes, and consequently, in LBW [125]. Both in humans and animal models, it has been demonstrated that a nutrient deficiency through the placenta, is associated with LBW and the development of CVDs in adulthood [127-129].

It is important to highlight that maternal undernutrition seems to be the major cause of LBW in underdeveloped or developing countries, while in high-income societies, LBW is mostly related with prematurity or obstetric complications associated with delayed pregnancy. For example, placental insufficiency and pre-eclampsia, which cause placental hypoxia and fetal oxygen and nutrient delivery privation [130, 131], are conditions related with the increase in maternal age [132, 133] and with offspring hypertension in adult life [133].

b. Other Fetal Stress Factors

In addition to an insufficient supply of nutrients, other stress factors, such as low oxygen, exposure to toxic substances, excess GC or environmental pollutants, can also affect fetal development, contributing to FPH [118, 119, 134].

Low oxygen access to the fetus is also associated with LBW and with programming of CVDs. Together with undernutrition, gestational hypoxia is the most clinically relevant fetal stress factor with important consequences for maternal health and responsible for offspring programming [135]. Low oxygen can be the consequence of placental insufficiency or of low hypobaric pressure, as occurs in high altitude pregnancies. In addition, exposure to maternal hypoxia during early gestation may have a profound influence on placentation. In fact, children born at high altitude are smaller and there is also higher incidence of preeclampsia [136]. The programming effects of oxygen deprivation during fetal life have also been demonstrated in experimental animals [111].

A third group of stress factor are toxic substances, which even in low concentrations are known to induce fetal programming. Maternal smoking during pregnancy has a number of well-known short-term adverse effects on offspring, such as shortened gestation and fetal growth restriction [117, 137]. There is also evidence that fetal exposure to maternal

smoking may also have long-term health consequences. Exposure to tobacco smoke in utero was associated with hypertension in offspring [138, 139]. Alcohol consumption during the gestational period has also been related to LBW and preterm birth [116]. In addition to these life-style related substances, exposure during intrauterine life to environmental pollutants, present in soil, water or air, has also been demonstrated to induce fetal programming [140].

Excess GC exposure to the fetus may also limit fetal growth [141] and program the cardiovascular and endocrine systems [142]. This is clinically relevant, since women with risk of preterm delivery are treated with CG to improve fetal pulmonary function and reduce mortality. Besides, maternal psychological stress may abnormally elevate cortisol levels [143]. The placental enzyme, 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), inactivates GC and thus protects the developing fetus from the deleterious effects of excess GC [144]. However, this enzyme can be reduced in adverse conditions, such as undernutrition or hypoxia [144, 145], favoring cortisol access to the fetus and contributing to FPH.

Finally, fetal overnutrition, due to maternal obesity or excess weight gain during gestation, is another stress factor important for FPH development. In fact, maternal obesity is associated with an increased risk for obstetric complications such as gestational diabetes mellitus, gestational hypertension, preeclampsia or pre-term delivery that can also contribute to FPH [146, 147]. Thus, *in utero* exposure to overnutrition programs the fetus for both metabolic disease later in life, and recent studies indicate that placental function may also be disrupted and contribute to long term health risk [148, 149].

iii. Experimental Animal Models

Experimental data gathered in animal studies have been very valuable to understand the relationship between adverse intrauterine environment and increased susceptibility to hypertension. Many of these mimic the maternal causes of LBW such as maternal undernutrition, maternal stress, alcohol consumption or maternal smoking whereas

others mimic complications during pregnancy that are linked to increased cardiovascular risk in the offspring such as preeclampsia or gestational diabetes [96, 150].

a. Animal Models of Fetal Undernutrition

As it was already said, reduced fetal access to nutrients could be induced by decreasing the mother's nutritional intake during the gestational period or by reducing placental blood flow. A variety of animal species, including the mouse, rat and sheep, have been used to study this effect.

Concerning the nutritional manipulation, the models employed differ, using various protein:lipid:carbohydrate ratios of the maternal diet during gestation, and varying timing and duration of dietary manipulation [151]. More specifically, the mother's diet can be modified by reducing the total caloric intake, the protein intake or some micronutrient in the diet, such as minerals or vitamins. Most of the FPH studies used a maternal general (caloric) undernutrition or a maternal low-protein diet animal models since both interventions resulted in significantly increased BP in the offspring [151]. General undernutrition (50 to 70% food restriction) during gestation in the rat programs a marked increase in BP associated with vascular dysfunction in the offspring in later life and, in mouse, programs an increase in BP associated with cardiac enlargement and an increase in coronary perivascular fibrosis [96]. Maternal protein restriction (8% versus 18%) during gestation programs an increase in BP in adult rat offspring, mouse, sheep or cow, indicating that the long-term impact of nutritional insults during fetal life on later CVDs is not species-specific [96, 152].

Animal models of reduced uteroplacental perfusion during late gestation, mimicking placental insufficiency, also lead to an environment of undernutrition and hypertension in the LBW offspring [112, 152]. This can be achieved through surgical models, being the most common the maternal bilateral uterine vessel ligation [153]. This model, when initiated at day 18 of gestation in the rat, programs hypertension in male offspring associated with vascular dysfunction [153, 154].

b. Sex-Differences

The majority of the experimental studies in these animal models have evidenced the important influence of sex in FPH. As so, male offspring develop hypertension by adult age, while females remain normotensive or develop milder forms of hypertension [155, 156]. These sexual dimorphism has been proposed to be related to the protective effects of estrogens [157-159]. Estrogen has been proposed as key factor that contributes to female protection against FPH, due its capacity to interact with the RAS system, its antioxidant properties, and the effects of estrogens on eNOS expression and activity, increasing vasodilation [160]. Besides, the role of testosterone has also been suggested, because animals exposed to placental insufficiency exhibit higher levels of testosterone compared to controls, and castration completely abolished hypertension in male offspring with LBW [159]. The contribution of sex steroids in the developmental programming of hypertension may involve modulation of neurohormonal systems, particularly RAS [158-160].

In addition to the influence of sex steroids on the sexual dimorphism of FPH, the concept of sex of the placenta has also been put forward, and it has been suggested that the placenta nourishing female fetus could adapt better to adverse intrauterine environments, thus, contributing to the lower impact of FPH in females [161, 162].

Despite the fact that in animal models of FPH the role of sex has been well documented, in humans, this is not so evident. This discrepancy is likely related to differences in the age of the populations under study, and the confounding factor of menopause [156, 157].

iv. Possible Mechanisms Implicated In Fetal Programming

In the above experimental animal studies some mechanisms have been pointed out to contribute to hypertension development in the offspring. Among them, high BP is associated with alterations in blood vessels (remodelling, stiffening and endothelial dysfunction), heart (cardiomyocyte hypertrophy or intramyocardial fibrosis), and deficient renal activity (low nephron number, alterations in sodium handling, etc.) [101, 163-166]. In addition, increasing evidence has shown the implication of oxidative stress

[167, 168], RAS [169-171] and SNS [172-174], as well as alterations in the GC axis [101, 113, 175, 176]. In the figure below, Figure 14, the proposed mechanisms underlying FPH that are most relevant for this work, are summarized.

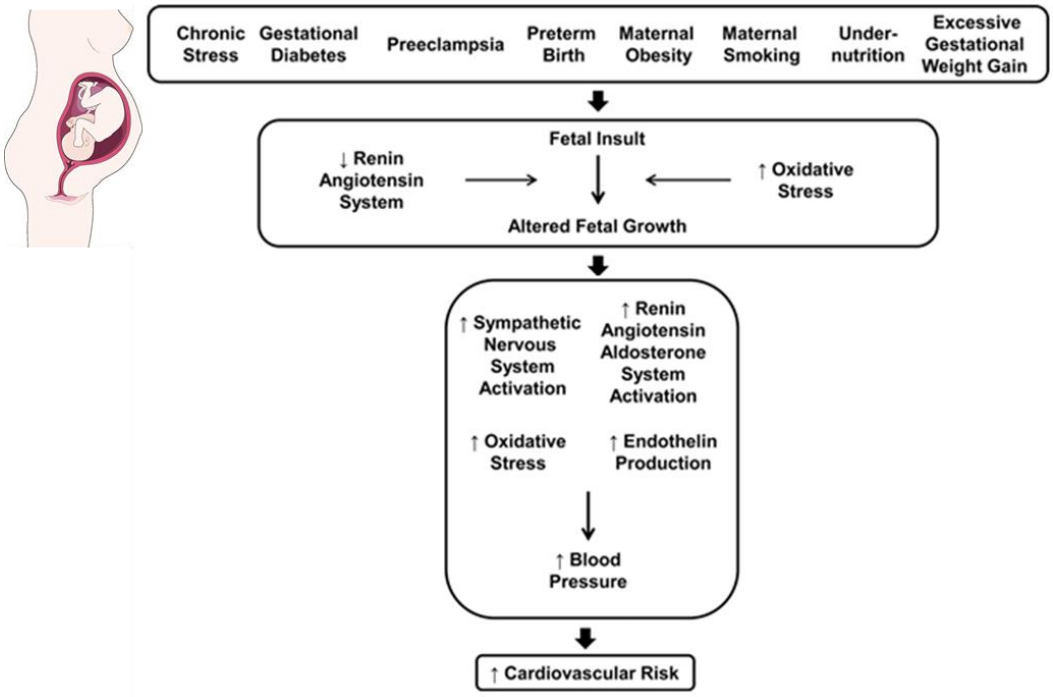

FIGURE 14| Schematic diagram of the mechanisms implicated in fetal programming of cardiovascular diseases. Adverse exposure to maternal insults during gestational life programs alterations in structure and physiology that contribute to the development of increased cardiovascular risk in later life (adapted from Alexander *et al*., 2015).

a. Sympathetic Nervous System Hyperactivation

Several lines of research, in humans and experiment animal models, point at the SNS as an important contributor to FPH.

Numerous studies indicate that SNS activation is increased in LBW individuals [177-179]. In addition, increases in circulating catecholamines indicative of enhanced SNS activation are observed in LBW individuals and also in different experimental models of LBW [174, 180, 181].

Alterations in SNS control are also evidenced in several animal models of FPH, where hypertension is associated with increased cardiovascular sympathetic tone [111, 182-

184] and sympathetic hyperinnervation [173]. In addition to alterations at the peripheral level, data also indicates changes in central SNA [175, 183, 185].

Excess cortisol levels may also contribute to FPH through the SNS. For example, in sheep fetuses, it has been shown that adrenal GC contribute to cardiovascular regulation by modulation of SNA and arterial baroreflex [186].

Taken together, data are consistent with a pathophysiological role of the SNS in FPH, with evidence indicating the occurrence of an increased SNA accompanied by morphological alterations.

b. Alterations in the Renin-Angiotensin System

RAS alterations are one of the common findings in different models of FPH. However, in humans exposed to adverse intrauterine conditions, information is scarce regarding the RAS. Nevertheless, it was reported that children born from preeclamptic pregnancies have increased circulating levels of aldosterone, a mineralocorticoid hormone which secretion is controlled by Ang II [187]. Also, boys with LBW exhibited a significant increase in ACE activity and circulating Ang II, which were associated with increased systolic BP [188]. However, this association was not found in girls, supporting the existence of sex differences in FPH also in humans. Indeed, RAS alterations associated with FPH seem to be age and sex-specific [156, 158].

Alterations of angiotensin receptors to FPH has been demonstrated in animals exposed to nutritional insults such as low-protein, high-fat or sugar diets [189-191] and toxics, such as nicotine [192, 193], or to CG [158]. ACE is other RAS player modified in FPH. Both pulmonary and plasma ACE activities seem to be increased in MUN adult rat offspring [194]. The role of Ang II in experimental FPH, hypertension is further demonstrated by evidence that BP rise is controlled after treatment with ACE inhibitors or Ang II receptors antagonists [170, 194, 195] similarly to what occur in essential hypertension.

Overall, experimental data are consistent and support the idea that RAS might be a common mechanism that underlies hypertension of developmental origins.

c. *Oxidative Stress*

Oxidative stress, defined as the imbalance between free radical producing systems and antioxidant defence systems of the body, is closely associated with inflammation [196], cardiovascular fibrosis and stiffness [31, 197] as well as endothelial dysfunction [14].

It is plausible that it might be the common denominator of cardiovascular changes in FPH. Some data support this hypothesis: i) placental alterations associated with fetal stress generates ROS linked to ischemia-reperfusion that might affect the development and function of the fetal vessels [198]; ii) diets rich in antioxidants during gestation and lactation in rats confer cardiovascular protection in adults [199-203]; iii) elevated BP and vascular dysfunction are characterized by enhanced vascular superoxide anion production that is generated, at least in part, by NADPH oxidase in FPH animals models [112].

Numerous data suggest that oxidative stress is at the basis of obstetric and fetal complications associated with LBW and also an underlying mechanism in FPH. During pregnancy, ROS exert important functions in placentation and serve as a signalling molecule, allowing the normal progression of embryonic and fetal development. Since embryo and fetus have low antioxidant capacity, an excess production in ROS during the intrauterine period would lead to a pro-oxidative state compromising fetal growth [168]. Furthermore, oxidative stress is also at the basis of changes in the kidney, heart, blood vessels and cardiovascular control systems. Particularly in vessels, oxidative stress contributes to endothelial dysfunction, observed in animal models of fetal programming [96, 150]. Reduced NO bioavailability caused by superoxide anion is one of the alterations consistently found in animals exposed to various fetal stress factors, namely nutrient deficiency, hypoxia, excess GC, or placental insufficiency [168]. Another factor is the reduced antioxidant capacity, also detected in FPH animals [168, 204].

Interestingly, the presence of oxidative damage prior to BP elevation, supports a role of redox disbalance as a causative element implicated in FPH [204].

d. Epigenetic Modifications

Epigenetics is the study of molecular processes occurring on and around the genome that regulate gene activity without changing the underlying DNA sequence [205]. Accordingly, epigenetic changes modify the expression of specific genes without changing the sequence of an individual's genome, and can be transmitted across generations.

Epigenetics describes the micro-structure of DNA and its associated proteins, which may be modified to induce upregulation or downregulation of specific genes, by three closely related mechanisms: DNA methylation, chromatin remodelling, and histone modification [206]. Several studies implicate these mechanisms in fetal programming and in the transmission of alterations to future generations [168, 206], particularly implicating the RAS and oxidative stress.

Considering the RAS, it had been demonstrated a methylation and an upregulation of mRNA and protein expressions of AT_1 receptor gene associated with histone modifications in blood vessels of different animal models of FPH [207, 208]. In addition, in brains of FPH animal models was reported an upregulation of mRNA expression of angiotensinogen and ACE and a downregulation of mRNA levels of AT_2 receptor [209]. Recently, it was presented the hypothesis that the expression of RAS components in the heart and VSMC might be induced by epigenetic factors activated during pregnancy [210]. This activation lead to the expression of renin and Ang II inside the cardiac and VSMC with consequent decrease of cell communication, impairment of metabolic cooperation, and changes in vascular smooth muscle contractility and dysfunction.

Other epigenetic modifications demonstrated in the context of FPH are the hypomethylation of eNOS promoter found in human umbilical endothelium from LBW fetuses and the alterations in histone deacetylation processes [168].

IV. MESENTERIC CIRCULATION

The branches of the abdominal aorta are divided into parietal and visceral parts. The visceral arteries are in turn divided into paired and unpaired branches. There are three major unpaired branches: the celiac trunk, the superior and the inferior mesenteric artery. Each has several major branches supplying the abdominal organs: the superior mesenteric artery supplies the pancreas, small intestine and the colon, whereas the inferior mesenteric artery supplies the descending colon and rectum (Figure 15) [17].

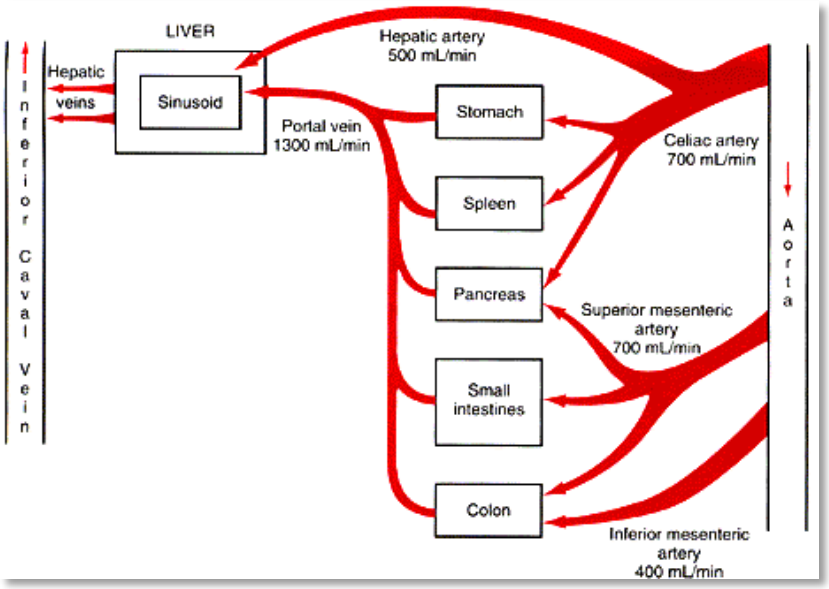

FIGURE 15| Schematic representation of splanchnic circulation. (From McGuire et al., 1993).

The mesenteric circulation plays an important role in the maintenance of systemic BP, and regulation of tissue blood flow [211]. Actually, the entire splanchnic circulation can receive up to 60% of cardiac output and contains about one third of the total blood volume. Mesenteric arteries and veins have significant resistance and capacitance functions in the systemic circulation, respectively. In comparison to the mesenteric vein, mesenteric artery has a high resting basal tone mediated in part by a thicker layer of VSMC [212]. Constriction of the mesenteric artery is thought to increase total peripheral resistance in the systemic circulation. In contrast, the mesenteric vein contains fewer layers of VSMC and is a more compliant vessel. The function of these low pressure vessels is to store significant quantities of blood that can be utilized to maintain the central venous pool of blood and cardiac output.

Sympathetic nerves innervating the splanchnic circulation are of upmost importance [213]. Mesentery arteries and veins are densely innervated by sympathetic nerve endings which play a critical role in the regulation of peripheral resistance and capacitance, respectively [214-217]. Indeed, when an increase in venomotor tone occurs in mesenteric vein, an increase in venous return/cardiac output also occur having a high relevance on hemodynamic [214].

It is known that increased SNA to the splanchnic circulation may represent a common stage in the development of hypertension [213] . Various animal models of hypertension support the concept that vascular resistance changes in the splanchnic organs are similar in direction and magnitude to pressure changes. These resistance changes appear to result from increased responsiveness of the arterioles to a variety of constrictor influences, and they may result from either structural or functional changes [218].

Hypothesis and Aims

BASIS AND HYPOTHESIS OF THE STUDY

- Hypertension is the primary risk factor for CVDs, a serious worldwide health concern and a significant healthcare burden.
- Exposure to adverse fetal environments, leading to LBW, predisposes to hypertension development, being males more susceptible. This process is known as fetal programming of hypertension.
- SNS and RAS hyperactivation and oxidative stress are common denominators in hypertension development. There is evidence of alterations in these systems associated with fetal programming. However, the relationship between these systems in the vascular wall have not been fully elaborated.
- The classic conceptualization of the RAS has undergone significant revision with the discovery of new active peptides and receptors. Their role of in fetal programming remains largely unknown.

Based on the above assumptions, the following **hypothesis** is postulated:

Suboptimal nutrition during fetal life induces hypertension in male rats, at least in part, through an alteration in vascular sympathetic neurotransmission, and cross-talk with RAS (Figure 16).

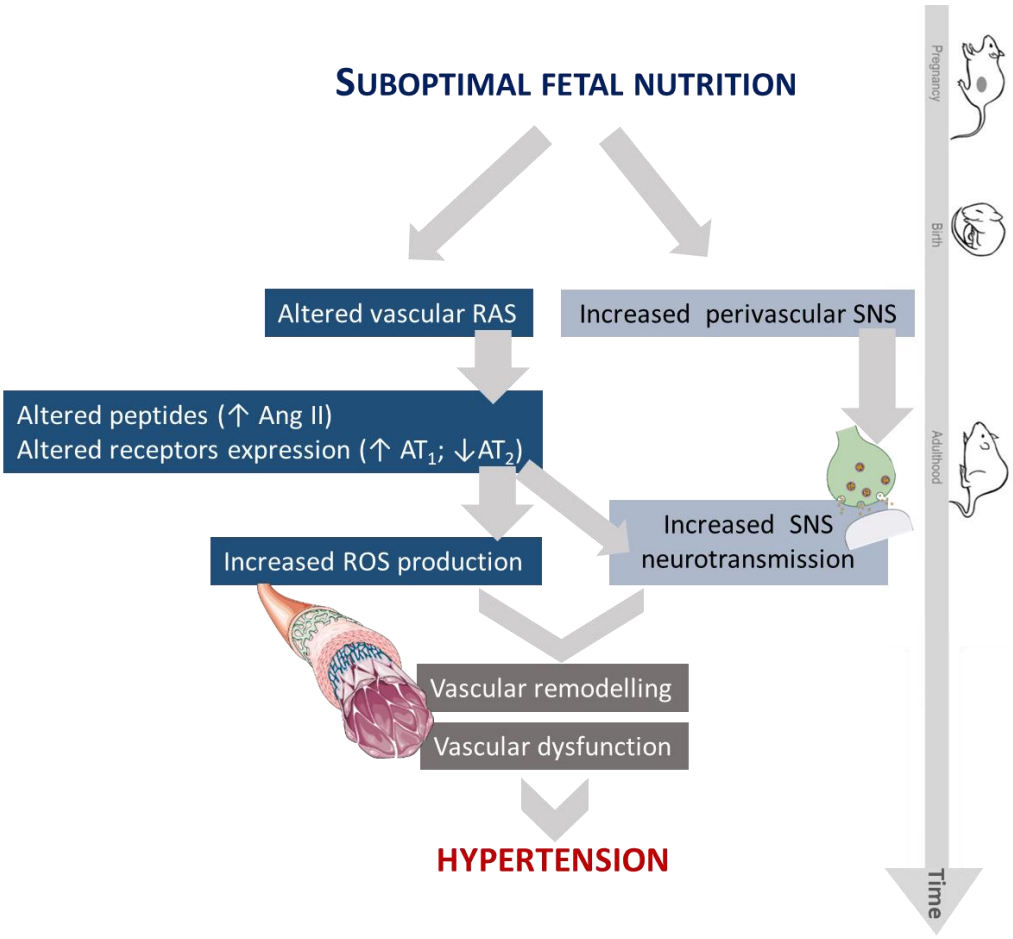

FIGURE 16| Schematic representation of the hypothesis of this Thesis.

AIMS

The **general aim** of this thesis is to analyse if exposure to suboptimal nutrition during fetal life in rats induces alterations in vascular sympathetic neurotransmission and RAS, which may be implicated in hypertension development. To achieve this, an experimental rat model of fetal programming based on maternal undernutrition during gestation was used and male offspring at adult age were studied. Another aim of this work is to compare vascular alterations in sympathetic neurotransmission induced by fetal undernutrition and those present in essential hypertension. For this comparison SHR were used.

Specific aims:

i. **To evaluate the influence of fetal undernutrition on hemodynamic parameters:**
    a. To analyse BP and HR in adult male rats exposed to fetal undernutrition.
    b. To compare the alterations in hemodynamic parameters induced by fetal undernutrition with those present in the SHR, a model of essential hypertension.

ii. **To assess the influence of fetal undernutrition on mesenteric artery remodelling** :
    a. To determine morphological alterations
    b. To determine alterations in collagen content
    c. To compare the alterations induced by fetal undernutrition and essential hypertension.

iii. **To assess the influence of fetal undernutrition on vascular sympathetic neurotransmission in the mesenteric artery:**
    a. To determine alterations in sympathetic innervation.
    b. To determine alterations in NA release and uptake.
    c. To compare alterations induced by fetal undernutrition and essential hypertension.

iv. **To assess the influence of maternal undernutrition on RAS**
    a. To assess alterations in plasmatic concentration of Ang II.
    b. To assess alterations in the expression of the main enzymes and receptors of the RAS in mesenteric arteries.
    c. To determine the location of RAS receptors in adventitial cells and sympathetic innervation.

v. **To determine the possible influence of RAS on sympathetic neurotransmission**
    a. To assess the influence of Ang II on perivascular NA release and the receptors implicated.

vi. **To assess the influence of maternal undernutrition on vascular $\bullet O_2^-$ production.**
    a. To assess alterations in adventitial NADPH oxidase, responsible for ROS production

Materials and Methods

I. EXPERIMENTAL ANIMAL MODELS

i. ETHICAL CONSIDERATIONS

In this thesis two different experimental animal models were used; a rat model of FPH and a rat model of essential hypertension, the SHR. The rats used were from the colony maintained at the animal house facility of the Universidad Autónoma de Madrid (UAM). All of the experimental procedures were approved by the Ethics Review Board of UAM (CEI63-1112-A097) and Comunidad Autónoma de Madrid (PROEX 199/15), and conformed to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised in 1996), the Spanish legislation (RD 1201/2005) and the Directive 2010/63/EU on the protection of animals used for scientific purposes.

ii. ANIMAL MODEL OF FETAL PROGRAMMING OF HYPERTENSION

In this work was used an animal model of FPH based on maternal undernutrition during gestation in Sprague Dawley rats (MUN), as previously described [204, 219]. The rats were mated in a cuvette with a grid bottom on filter paper. The morning after the day of mating, the presence of a mucous vaginal plug on the floor of the cuvette indicated day 1 of gestation. At that time, the female was separated from the male and was housed individually in a bucket on aspen wood, with the following dimensions: 36.5 cm long, 21.5 cm wide and 18.5 cm high. The buckets were placed in rooms under controlled conditions with regulated temperature (19-23 °C), controlled relative humidity (35-45%) and 12/12 light/dark photoperiod. The animal health monitoring indicated that the rats were free from pathogens that may interact with any of the studied parameters.

All the rats were fed with breeding diet (SAFE A03; Safe Augy, France) of the following composition: 51.7% carbohydrates, 21.4% protein, 5.1% lipids, 3.9% fiber, 5.7% minerals and 12.2% humidity. Drinking water was supplied *ad libitum* in all cases.

Two study groups were established: a control group, with *ad libitum* feeding throughout pregnancy and lactation and a group with intake restriction during part of gestation. This last group of rats had *ad libitum* diet during the first half of the gestation (from day 1 to 10) and then, they were fed with 50% of the intake of a pregnant rat (from day 11 until delivery). To determine the amount corresponding to the 50% of the normal intake, preliminary studies were carried out. These experiments showed that the maximum intake of a pregnant rat was 24 g/day. After delivery and through all the lactation period the mothers were fed *ad libitum*.

Immediately after birth, the offspring was weighed individually and sexed, and the litter was standardized to 12 rats, 6 males and 6 females if possible. The rest of the litter was sacrificed with CO_2. At the age of 6 months, male offspring from the two experimental groups were analysed: from rats exposed to maternal undernutrition during pregnancy (MUN) and from control rats with mothers fed *ad libitum* during pregnancy (CONTROL). Rats from at least 3 different litters were used for each experimental protocol. A schematic diagram showing the experimental model is shown in Figure 17.

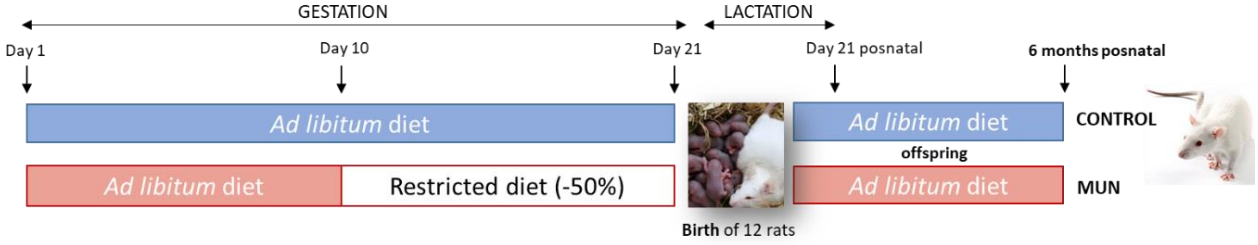

FIGURE 17| Experimental animal model of fetal programming of hypertension based on maternal undernutrition.

iii. ANIMAL MODEL OF ESSENTIAL HYPERTENSION

In the present work the SHR were wealso used, a well-known animal model of essential hypertension to make comparisons with the animal model of FPH induced by MUN. Since the SHR have been created by selective breeding of Wistar-Kyoto rats (WKY), the WKY was chosen as the control model, representing a normotensive state. Both WKY and SHR were bred at the Animal House of Universidad Autónoma de Madrid.

II. *IN VIVO* EXPERIMENTAL PROCEDURES

i. HEMODYNAMIC PARAMETERS IN ANESTHETIZED RATS

Intra-arterial BP was assessed as previously described [220]. Briefly, the rats were anaesthetized with 37.5 mg/kg Ketamine hydrochloride and 0.25 mg/kg Medetomidine hydrochloride i.p. and placed on a heating blanket. The iliac artery was exposed and a catheter was inserted (filled with 0.9% saline solution with 1% heparin). The catheter was connected to a pressure transducer (Statham; Harvard Apparatus) and the transducer to a PowerLab system/8SP (ADInstruments), where the pressure wave was displayed. After cannulation the pressure was recorded for approximately 60 min and stored for later analysis of data. HR, DBP and SBP were measured in the chart, averaging over approximately 1 min of the last part of the recording period (Figure 18).

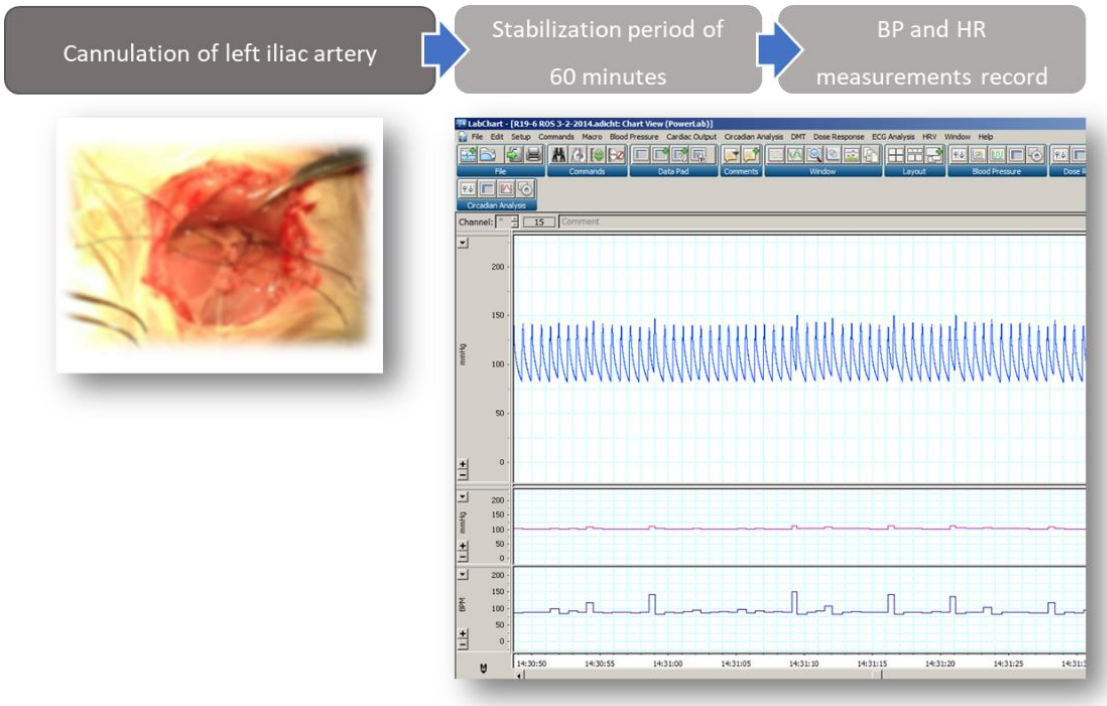

FIGURE 18| Schematic representation of *in vivo* blood pressure measurement. After 60 minutes of stabilization, the blood pressure and heart rate were analysed from the chart.

ii. TAIL-CUFF PLETHYSMOGRAPHY

To evaluate hemodynamic parameters without the effect of anaesthesia the tail-cuff plethysmography was used. BP was analysed with a NIPREM 645 non-invasive BP acquisition system for rats (CIBERTEC, Madrid, Spain). The experiments were conducted in a quiet area at 22±2°C. Firstly, the rats were placed in a chamber at 37 °C for 10-15 min to induce vasodilatation. Thereafter, they were placed inside a soft support in the darkness to prevent excessive movement. A pulse sensor and a pressure cuff were place around the tail for BP recordings (Statham, Harvard Aparatos GMBH, Germany) (Figure 19). The cuff was inflated to 150 mmHg and 15 to 25 pressure inflate-deflate cycles were performed and data were automatically registered (PowerLab system-ADInstruments). The first 5 cycles were considered "acclimatization" cycles and were not used in the analysis. This procedure was obtained during 3 consecutive days for the rats to become accustomed. The measurements of SBP recorded on days 2 and 3 were averaged for statistical analysis.

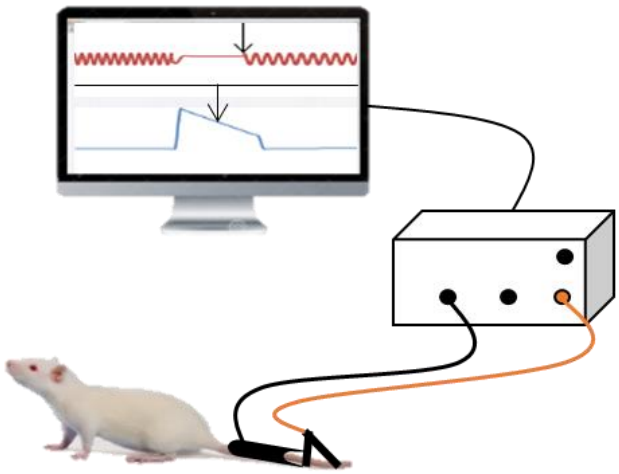

FIGURE 19| Example of systolic blood pressure measurement by tail-cuff plethysmography.

III. SAMPLE COLLECTION

The animals were first weighted and then anesthetized in a CO_2 chamber, and a blood sample was obtained by cardiac puncture using a syringe, and placed into EDTA-containing tubes. Thereafter, the blood was centrifuged (3000 rpm, 15 min, 4ºC) and the supernatant plasma was stored at -80ºC until used.

After the animals were euthanized by exsanguination the mesenteric bed and tail arteries were collected in cold Krebs–Henseleit solution (KHS) with the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03 (pH 7.4). Thereafter, the mesenteric artery and vein, and tail artery were dissected. For functional studies, these three vessels were used immediately. For histological studies, a small part of mesenteric artery from each rat was placed in paraformaldehyde 4% in phosphate buffer solution (PBS; in g/L: NaCl 8.0, $Na_2HPO_4.2H_2O$ 0.77, KCl 0.20, KH_2PO_4 0.19; pH 7.2) during 50 min for immunohistochemical procedures or, for other procedures, until used. For RT-PCR the mesenteric arteries were frozen with liquid nitrogen and stored at -80ºC until used.

IV. VASCULAR HISTOLOGY

i. CHEMICALS

For this study the following staining were used: hematoxylin-eosin (Merck cat. no. 1.09249.2500 and 1.15935.0025 Darmstadt, Germany), Masson's trichrome (Merck cat. no. 1.16316.0050 Darmstadt, Germany) to assess connective tissue components (collagen and muscle cells), and orcein (Merck cat. no. 1.07100 Darmstadt, Germany) to detect elastic fibres. Entellan (Merck cat. no. 1.07961.0500 Darmstadt, Germany) was used as a mounting medium.

ii. Experimental Histological Procedure

Briefly, the mesenteric artery placed in paraformaldehyde 4% PBS was dehydrated in a growing alcohol battery and rinsed in xylene. Thereafter, it was included in a paraffin block, and serial sections of 2 µm thickness were made using a microtome. The sections were dewaxed in xylene, then they were hydrated in alcohols of decreasing concentrations and stained with haematoxylin-eosin, Masson's trichrome and orcein.

Each tissue was cut in five levels along the length of the vessel to ensure data represents the putative mesenteric artery heterogeneity rather than only a specific location of the artery. Each batch represents histochemical staining for one type of dye and include sections from all five levels of mesenteric artery from each animal. This procedure was repeated three times (3 batches). In total, for each treatment, 150 sections were obtained. Sections were stained for each dye and divided according to animal source. Within each of these groups, a random selection of the sections was carried out.

iii. Image acquisition and quantification

Stained sections were visualized using a high-resolution Zeiss Axiocam 105 color digital camera mounted on a Zeiss Primo Star microscope, using an X10 objective, to analyse the arterial lumen, media, and adventitia layer. Morphometry was performed with Image J software [221], and data of lumen diameter, cross sectional area of the media and the adventitia and collagen content were obtained (Figure 20).

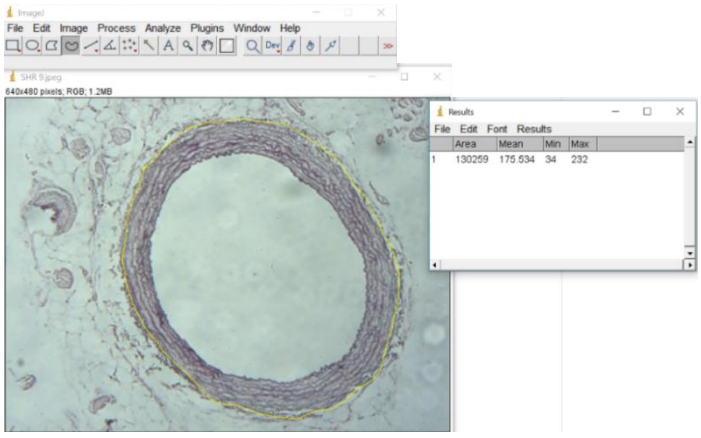

Figure 20| Example of morphometry analysis using ImageJ. Section of a mesenteric artery: in yellow it is marked the external perimeter of the media; in the right panel the total area of the artery is represented.

V. PRESYNAPTIC FUNCTION

i. BACKGROUND

To study the vascular presynaptic function, [^{3}H]-NA was used, possible to trace, and electric field stimulation that simulates physiological conditions of an action potential. Indeed, electrically evoked tritium overflow from tissue preparations incubated with radioactive NA has been shown to reflect action potential-evoked neuronal NA release and drug-induced changes in evoked tritium overflow have been assumed to reflect changes in neuronal NA release, as observed in previous studies [26, 222, 223]. In this way, it is possible to study the receptors implicated in presynaptic function with specific drugs (agonists and/or antagonists). Additionally, it is possible to estimate the amount of NA that has been incorporated into the nerve terminal (Figure 21).

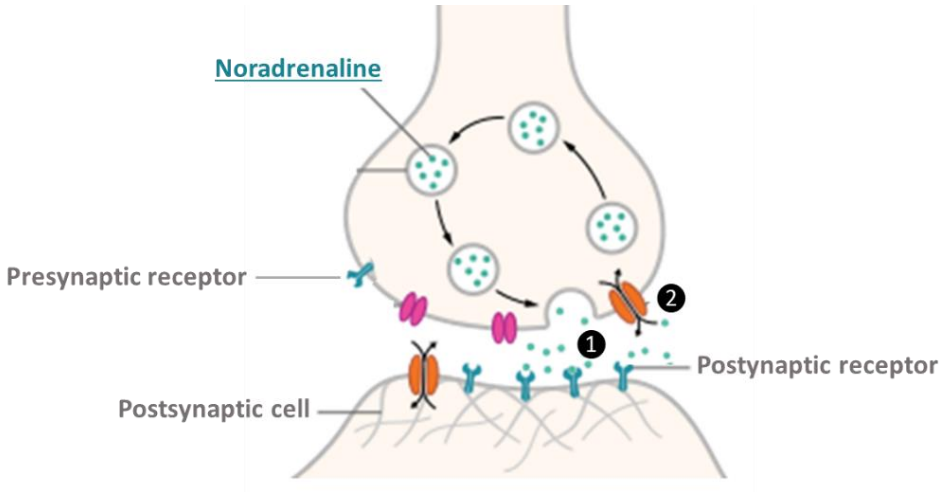

FIGURE 21| Schematic representation of NA release (1) and uptake (2) in the synaptic cleft.

ii. CHEMICALS

The following drugs were used: levo-[ring-2,5,6-3H]-noradrenaline, specific activity 44.8 Ci/mmol (DuPont NEN, I.L.C., Lisboa, Portugal), losartan (Merck Portuguesa, Lisbon, Portugal), angiotensin II (Sigma-Aldrich, St. Louis, USA) and desipramine hydrochloride purchased from Sigma-Aldrich (Sintra, Portugal). Stock solutions were made up in ultra-

pure water and diluted in superfusion medium immediately before use. The scintillation mixture used was from OptiPhase 'Hisafe' 3, PerkinElmer, I.L.C., Lisboa, Portugal.

iii. Experimental Procedure

[^{3}H]-NA release experiments were carried out as previously described [26, 224, 225]. Vessels were preincubated in 2 mL KHS containing 0.1 µmol/L [^{3}H]-NA (for 60 min at 37°C) and transferred into superfusion chambers, superfused with [^{3}H]-NA free medium of KHS (1 mL/min; constant rate) with desipramine 400 nmol/L to inhibit NA's neuronal uptake. Three identical periods of electrical stimulation were applied (Hugo Sachs Elektronik, March-Hugstetten, Germany; constant current mode, rectangular pulses; 1 ms, with current strength 50 mA; 5 Hz, 100 pulses). The first, starting at t=30 min (S_0) was not used for determination of tritium outflow. The subsequent periods (S_1 and S_2) were applied at t=90 min and t=120 min, respectively. The superfusate was collected each 5 min period, starting from minute 85 of superfusion onwards.

Implication of presynaptic AT_1 receptors was addressed using an agonist for these receptors, Ang II (30 nmol/L), and an antagonist, Losartan (100 nmol/L): Ang II was added 5 min before S_2 and kept until the end of the stimulation period; losartan was added immediately after S_1 and kept until the end of the experimental procedure (Figure 22).

At the end of experiments (t=130 min), tritium was measured in the collected superfusate samples and in the vessels (previously solubilized: sonicated 1 h with 2.5 mL, 0.2 mol/L perchloric acid). For this, 6 mL of a scintillation mixture was added to each sample and, thereafter, analysed by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA).

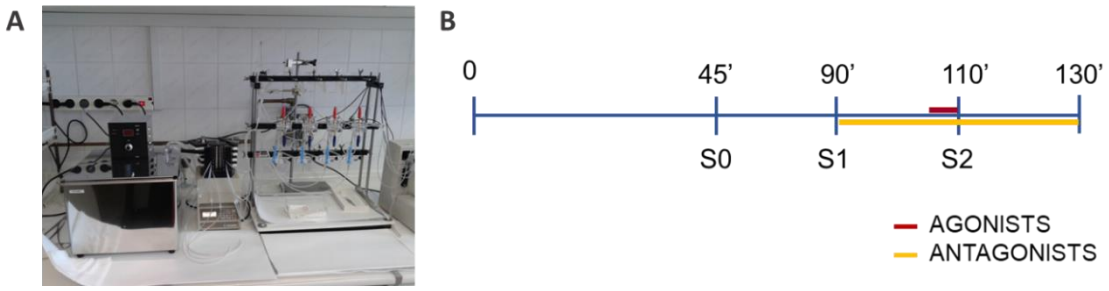

FIGURE 22| Superfusion chambers (A) and schematic representation of [^{3}H]-NA release experiments (B). Tissue preparations were pre-incubated with [^{3}H]-NA for 60 min. After that, tissues were superfused with [^{3}H]-NA free medium containing desipramine and were stimulated three times at 30-min intervals (S_0, S_1 and S_2). The superfusate was collected each 5 min period from minute 85 of superfusion onwards. Antagonists (yellow line) added immediately after S_1 and kept until the end of the experimental procedure; agonists (red line) added 5 min before S_2 and kept until the end of the stimulation period.

iv. QUANTIFICATION

Tissue labelling with [^{3}H]-NA and evaluation of electrically-evoked tritium overflow changes was performed as previously described [26, 224, 225]. Effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as the ratio of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments in which a drug was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of the respective drug).

Values of [^{3}H]-NA uptake were estimated using the following approach. At the end of each [^{3}H]-NA release experiment, the tissue tritium content was obtained. This content was sum to values of [^{3}H]-NA previously collected in the 5-min superfusate samples (from t = 85 min to t = 130 min, control preparations). The sum final value was considered to be the total amount of incorporated [^{3}H]-NA in individual mesenteric artery preparations (total tissue tritium content).

VI. REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

i. BACKGROUND

RT-PCR is a technology by which RNA molecules are converted into their complementary DNA (cDNA) sequences by reverse transcriptases, followed by the amplification of the newly synthesized cDNA by standard PCR procedures [226]. The cDNA is then used as the template for the quantitative PCR reaction (Figure 23). In this work, this technique was used to quantify RAS receptors (AT_1, AT_2, Mas and MrgD) and enzymes (renin, ACE and ACE 2).

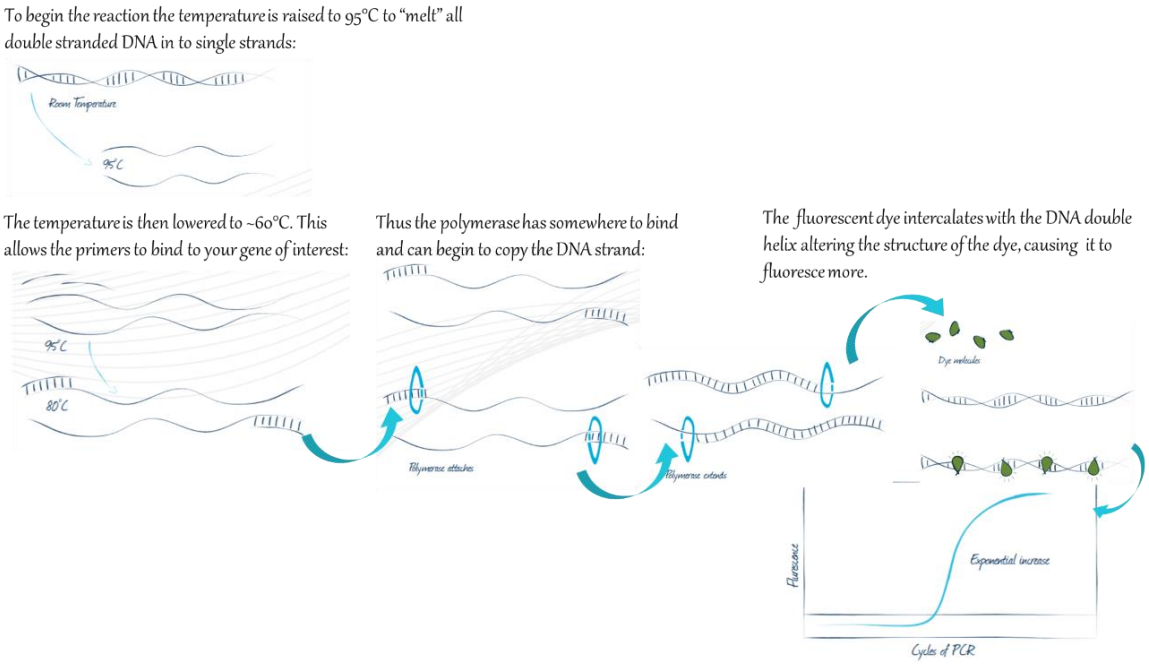

FIGURE 23| Basic principles of reverse transcriptase polymerase chain reaction. Adapted from "*Beginner's Guide to Real-Time PCR*", Primerdesign, UK.

ii. CHEMICALS

Specifically, for the extraction procedure RNA*later*®-ICE (Thermo Fisher, US) and TRIzol (Invitrogen, ThermoFisher Scientific, USA) were used. The Kit used for cDNA synthesis was Xpert cDNA Synthesis Mastermix Kit (GRiSP, Porto, Portugal) and the fluorophore for RT-PCR was 1X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Milan, Italy). The primers used are mentioned in the Table 1.

TABLE 1| Reference and target genes: primer specifications

Gene	Primer Sequence (5' → 3')	Lenght (bp)	GenBank nº
Renin	F: CTAAAGCATCTCGCCAAGG R: ACCAGGGATGTGTCGAATGA		AB188298
ACE	F: TCCTATTCCCGCTCATCT R: CCAGCCCTTCTGTACCATT	127	NM_012544.1
ACE 2	F: GAATGCGACCATCAAGCG R: CAAGCCCAGAGCCTACGA	228	AY881244
AT_1 receptor	F: TCTGGATAAATCACACAACCCTC R: GAGTTGGTCTCAGACACTATTCG	77	NM_030985.4
AT_2 receptor	F: CTGGCAAGCATCTTATGTAGTTC R: ACAAGCATTCACACCTAAGTATTC	115	U22663.1
Mas	F: ACTGTCGGGCGGTCATCATC R: GGTGGAGAAAAGCAAGGAGA	272	NM_012757.2
MrgD	F: CAGCCTCGGCGGCTCTA R: CCAACGGCAGAGAACAGGTAAG		
Hprt-1	F: CCCAGCGTCGTGATTAGTGATG R: TTCAGTCCTGTCCATAATCAGTCC	110	NM_012583
Hmbs	F: TCTAGATGGCTCAGATAGCATGCA R: TGGACCATCTTCTTGCTGAACA	76	NM_013168
Papbn-1	F: TATGGTGCGACAGCAGAAGA R: TATGCAAACCCTTTGGGATG	110	116697

Primers selected for renin-angiotensin study, including the main enzymes (renin, ACE, ACE2) and receptors (AT_1, AT_2, Mas and MrgD). The 3 housekeeping genes used were: Hprt-1, Hmbs and Papbn-1. F, forward primer sequence (5'-3'); R, reverse primer sequence (5'-3').

iii. RNA EXTRACTION AND REVERSE TRANSCRIPTION

For RNA extraction, mesenteric arteries were dissected ensuring maximum protection of the RNA; they were stored in sterile tubes and frozen at -80ºC, until their use. To defrost the arteries, RNA*later*®-ICE was used to process the samples by common homogenization methodology to extract high quality RNA. Soaking frozen tissue samples overnight in RNA*later*®-ICE at –20°C eliminates the need to pulverize frozen tissue samples, because soaking in RNAl*ater*®-ICE changes the physical state of the tissue from brittle to pliable at –20°C, minimizing RNA degradation. After that, samples were placed in TRIzol, homogenized in Precelys (Bertin Technologies, France) and RNA was extracted by following the manufacturer's instructions. Briefly, after homogenate the samples, chloroform was added and the tube was mixed. After centrifugation for 15 min at 12000 × g at 4°C the mixture separates into a lower red phenol-chloroform, an interphase, and

a colourless upper aqueous phase, which contains the RNA. This aqueous phase was transferred to a new tube to which isopropanol was added, incubate for 10 min and then centrifuged for 10 min at 12000 × g at 4°C. Total RNA precipitate formed a white gel-like pellet at the bottom of the tube. After discarding the supernatant, the pellet were resuspended in 75% ethanol, mixed and centrifuged for 5 min at 7500 × g at 4°C. Finally the supernatant was discarded and the pellet was air dried for 10 min.

The pellet was resuspended in RNase-free water and incubated in a heat block set at 60°C for 15 min. The integrity of total RNA was detected by electrophoresis of samples on 1% agarose gel and total RNA purity and concentration were evaluated spectrophotometrically (Synergy HT MultiMode Microplate Reader, Biotek, VT, USA). The absorbance was measure at 260 nm and 280 nm and RNA concentration was calculated using the formula A260 × dilution × 40 = µg RNA/mL. The RNA purity was calculated by the A260/A280 ratio which is consider pure around 2.

The RNA samples were stored at −80◦C. A quantity of 1 µg of total RNA obtained from each sample was reverse transcribed with Xpert cDNA Synthesis Mastermix Kit according to the manufacturer's instructions.

iv. Quantitative RT-PCR Analysis of Gene Expression

Real-Time PCR reactions were carried out in a 96-well CFX RT-PCR System (Bio-Rad, Milan, Italy). Each reaction was performed 10 µl reaction mixture, including 2 ng of template cDNA, 0.4 µM of each primer and 1X SsoAdvanced Universal SYBR Green Supermix. Gene amplification protocol started with 95◦C for 30 s, followed by 39 cycles at 95◦C for 15 s and 60◦C for 30 s. Each assay was performed in triplicates, with negative control. The combination of GeNorm and qBase software technology, following recent guidelines, was used to choose 3 housekeeping genes: HPRT-1: hypoxanthine phosphoribosyltransferase I; Hmbs: Hydroxymethylbilane synthase; Papbn-1: Polyadenylate-binding protein 1 [227]. The average Ct values obtained from each triplicate was converted to a relative quantity and analysed with CFX Manager algorithm. Gene stability is expressed by the M value, which is calculated as the average variation between one of the housekeeping genes and all the others analysed. The

housekeeping genes chosen were considered stable for having the M value < 0.5. A standard curve for each target and housekeeping gene was evaluated to assess amplification efficiency and linearity.

VII. FLUORESCENCE CONFOCAL MICROSCOPY STUDY

i. BACKGROUND

To study simultaneously the location of RAS receptors on specific nerve structures, fluorescence confocal microscopy was used. Confocal microscopy allows to study thick tissues, due to the presence of a pinhole which discards the fluorescence coming from planes below and above the focal plane and permits the study of small structures with detail [13]. Other advantages over conventional optical microscopy, are the ability to control depth of field and the capability to collect serial optical sections from thick specimens.

ii. CHEMICALS

The following antibodies were used: rabbit polyclonal anti-AT_1 receptor (Santa Cruz Biotechnology, Inc., CA, USA); rabbit polyclonal anti-AT_2 receptor (Santa Cruz Biotechnology, Inc., CA, USA); rabbit polyclonal anti-Mas (Alomone labs, Jerusalem, Israel), rabbit polyclonal anti-MrgD (Alomone labs, Jerusalem, Israel); goat polyclonal anti-p22phox (Santa Cruz Biotechnology, Inc., CA, USA); mouse monoclonal anti-tyrosine hydroxilase (ab129991, Abcam, UK) and mouse monoclonal anti-glial fribrilary acidic protein (GFAP, G6171, Sigma-Aldrich, Inc., USA).

The following fluorescent probes were used: Alexa Fluor® 488 goat anti-mouse IgG (H+L) antibody, highly cross-adsorbed, Alexa Fluor® 594 anti-goat IgG (H+L) antibody, highly cross-adsorbed and Alexa Fluor® 647 goat anti-rabbit IgG (H+L) antibody, highly cross-adsorbed (Molecular Probes) secondary fluorescent antibodies (Invitrogen, Life

Technologies, SA, Madrid, Spain); vectashield mounting medium with DAPI (Vector Laboratories, UK).

iii. STAINING PROTOCOL

Immunohistochemistry procedures were previously described [222]. Briefly, the artery was immediately placed in cold PBS and was cut in 4 segments. Each artery segment was fixed (paraformaldehyde 4% PBS; 50 min; room temperature). After two 15 min PBS washing cycles, artery segments were incubated with the following primary antibodies:

- rabbit polyclonal antibodies against angiotensin receptors subtypes anti-AT_1 anti-AT_2, anti-Mas and anti-MrgD (1:100 dilution, overnight, 4ºC)
- goat polyclonal anti-p22phox (1:200 dilution, overnight, 4ºC) to stain NADPH oxidase.
- mouse monoclonal anti-tyrosine hydroxylase (TH, 1:100 dilution, overnight, 4ºC) to stain noradrenergic nerve terminals.
- mouse monoclonal anti-glial fibrillary acidic protein (GFAP, 1:200 dilution, overnight, 4ºC) to stain glial cells such as Schwann cells.

Thereafter, tissues were incubated with Alexa 647 anti-rabbit and Alexa 488 anti-mouse fluorescent secondary antibodies (1:1000 dilution, 1 h, room temperature). Negative controls were incubated on adjacent sections using 10% normal horse serum or blocking solution instead of the primary antibody. After three PBS washing cycles, the arteries were mounted intact with antifading agent (vectashield mounting medium with DAPI, Vector Laboratories, UK). A representative scheme of the protocol using a 96-well plate is depicted in Figure 24.

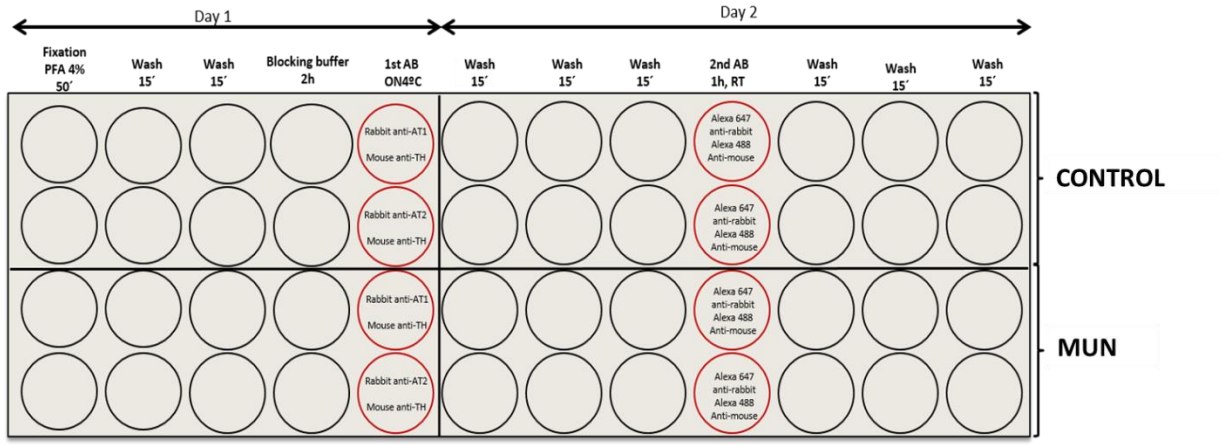

FIGURE 24| Representative scheme of immunohistochemistry experiments.

iv. LASER SCANNING CONFOCAL MICROSCOPY (LSCM)

Preparations were visualized with a Leica SP2 LSCM system (Leica Microsystems, Wetzlar, Germany) fitted with an inverted microscope (× 63 oil immersion lens) (Figure 25). Stacks of 1-µm-thick serial optical images were captured from 5 randomly chosen regions along the adventitial layer of the mesenteric artery, which was identified by the shape and orientation of the nuclei stained with DAPI [228].

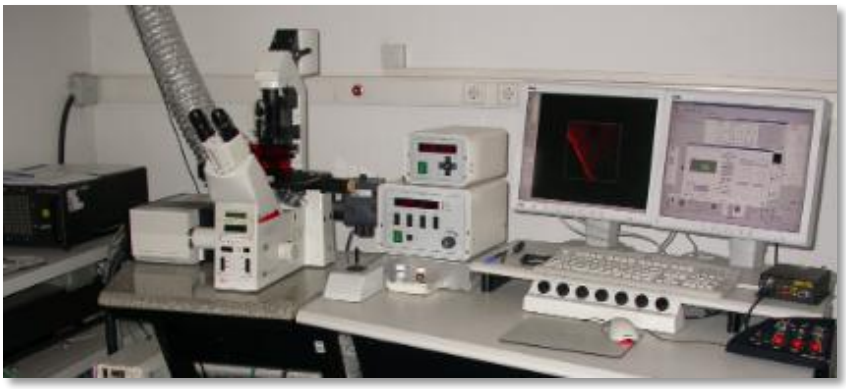

FIGURE 25| Confocal microscopy - Leica SP2 LSCM system of UAM.

From each region, three stacks of images were sequentially obtained at different wavelengths. The first stack was taken with the Ex 405 nm and Em 412-470 nm wavelength to visualize cell nuclei (DAPI staining). The second was taken with the Ex 488 nm and Em 490-570 nm wavelength to visualize the TH or GFAP staining (location of

noradrenergic terminals or glial cells) and the third with the Ex 633 nm and Em 640-720 nm wavelength, to detect RAS receptors distribution stained with the secondary antibody Alexa Fluor 647 (different subtypes depending on primary antibody). Image acquisition was performed always under the same laser power, brightness, and contrast conditions. The resulting images were reconstructed separately for each wavelength for later quantification.

v. Image Quantification

Quantitative analysis of confocal z-stacks images was performed using image analysis software (PAQI, CEMUP, Porto, Portugal), as previously described [229]. Briefly, a sequential routine was designed and developed to analyse each fluorescent signal used. PAQI software measured the surface area and strength of the fluorescence signal marking the postganglionic nerves, the surface area and strength of the attachment signal marking the receptors and determined the surface area of interception of the receptors on the nerves as well as the intensity of fluorescence of the receptors on nerves (corrected for background) (Table 2).

TABLE 2| Description of the algorithm steps for the quantification of AT_1 receptors, sympathetic nerves and of the interception of receptors and nerves

Step 1 *(analysis of sympathetic nerves) and* ***step 2*** *(analysis of AT_1 receptors)*
1. Ensure that image is properly prepared, acquired, and processed. 2. Open fluorescence images of sympathetic nerves or of AT_1 receptors. 3. Determining/Setting the threshold intensity reference background/noise (for all images). 4. Obtaining binary image that marks sympathetic nerves or AT_1 receptors. 5. Obtaining new image containing only the demarcated areas of sympathetic nerves or AT_1 receptors and corrected intensity background. 6. Sympathetic nerve or AT_1 receptors binary images analysis and determination of the surface area of the sympathetic nerves or surface area of attachment of the AT_1 receptors and the fluorescence intensity on the surface of the sympathetic nerves or AT_1 receptors, corrected for background.
Step 3 *(analysis of the receptors on the sympathetic nerves (overlay))*
1. Measurement of the surface area and strength of AT_1 receptors on the nerves. 2. Results: Fluorescence images of AT_1 receptors; dividing binary images of sympathetic nerves; dividing binary image AT_1 receptors. 3. Obtaining image that marks the AT_1 receptors on sympathetic nerves. 4. Obtaining image containing only the demarcated areas of AT_1 receptors on sympathetic nerves and corrected intensity background. 5. The latter is analysed for determination of the surface area of attachment of the AT_1 receptors on the sympathetic nerves and intensity of fluorescence of the AT_1 receptors on sympathetic nerves, corrected for background.

Adapted from JB Sousa *et al*., Microscopy: advances in scientific research and education, 2014.

VIII. PLASMA ANGIOTENSIN II

i. BACKGROUND

Enzyme-linked immunosorbent assay (ELISA) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In this technique the antigen must be immobilized on a solid surface and then complexed with an antibody linked to an enzyme. The detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product (Figure 26). A key element of the detection strategy is a highly specific antibody-antigen interaction.

In the present work this technique was used to quantify plasmatic Ang II from MUN and CONTROL rats.

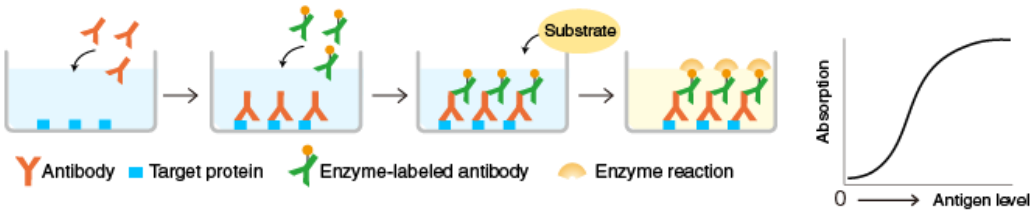

FIGURE 26| Basic principle of ELISA. A capture antibody on a multi-well plate will immobilize the antigen of interest. This antigen will be recognized and bound by a detection antibody conjugated to biotin and streptavidin-HRP.

ii. EXPERIMENTAL PROCEDURE

Ang II was first extracted from plasma samples by solid phase extraction (Discovery® DSC-Ph SPE tube, Sigma-Aldrich) (Figure 27). Briefly, the columns were activated with a sequence of 5 ml of the following solvents in this order: methanol, tetrahydrofuran, hexane, methanol and ultrapure water. Thereafter, 1 ml of plasma samples were placed in the columns and the extraction was made with another sequence of 5 ml of the following solvents in this order: ultrapure water, acetic acid 4% and finally a mixture consisting in ethanol:acetic acid:water (90:4:6). The last 5 ml with the mixture were collected, evaporated with nitrogen, followed by lyophilisation and stored at -80ºC until used.

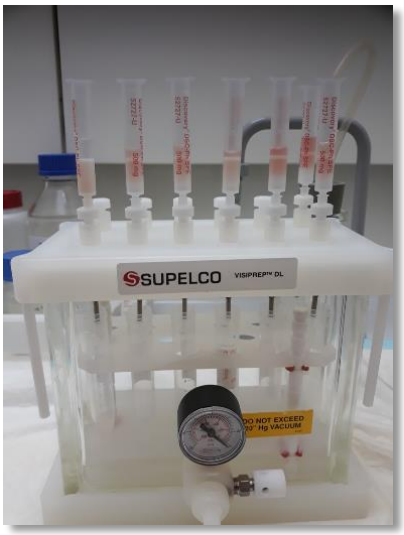

FIGURE 27| Solid phase extraction.

Ang II levels were quantified by ELISA using a commercial kit (Peninsula Laboratories International) according to the manufacturer's instructions. Briefly, in a 96-well plate, each sample and standard were loaded in duplicate and, after reaction, absorbance was read at 450 nm on a plate reader (Synergy HT MultiMode Microplate Reader, Biotek, VT, USA), within 10 min after the end of the protocol. The concentration of Ang II in the sample is inversely proportional to the measured optical density. For the calculation of results, the mean absorbance values of each duplicate of the sample were obtained and the concentration was determined from the standard curve.

IX. STATISTICAL ANALYSIS

Statistical analyses were performed with Graph-Pad Prism (version 8.3).

Sample size was calculated assuming a probability error of alpha type of 5% (p<0.05) and potency of 80%.

The normality of the variables was evaluated with Kolmogorov-Smirnov test. Since the variables followed a normal distribution, parametric tests were used. Results were expressed as mean±s.e.m. Differences of means were compared using one- or two-way ANOVA, followed by post-hoc Holm-Sidak's multicomparison *t* test or Student's *t* test. A *p* value lower than 0.05 was considered to denote statistically significant differences.

Results

I. HEMODYNAMIC PARAMETERS

At the age of 6 months, body weight was not significantly different between MUN and CONTROL male rats (MUN=460,18±8,1 g; CONTROL= 486,6±18,6g; *n*= 6; *p*=0,21). Statistical differences were not detect between body weight of SHR and WKY rats at the age of 6 months (SHR=377,4±8,9 g; WKY= 367,0±11,9 g; *n*=6, *p*=0,52).

Hemodynamic parameters were measure in rats under anaesthesia and in awake rats. In anaesthetized rats, MUN rats exhibited significantly larger SPB, DBP but not HR compared to their respective controls (Figure 28A and 29). SHR exhibited significantly larger SBP, DBP and HR compared to controls (Figure 28B and 29). MUN rats exhibited lower SBP and DBP levels compared to SHR rats.

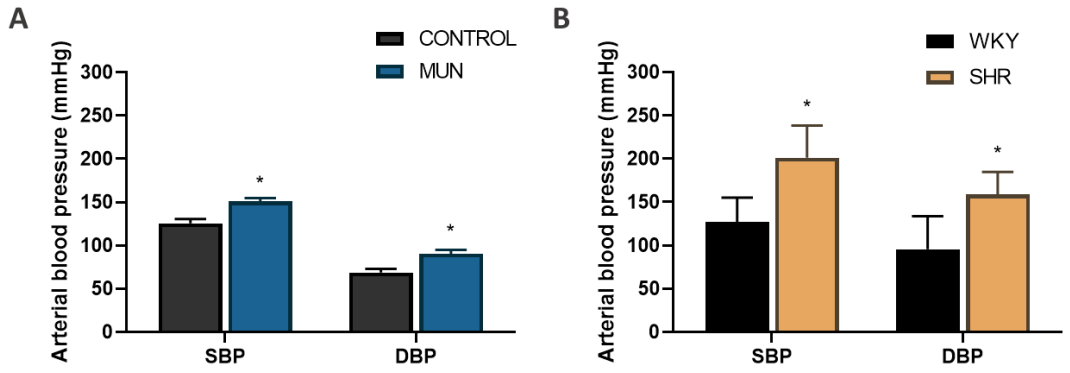

FIGURE 28| Systolic (SBP) and diastolic (DBP) blood pressure of the 4 animal groups in study. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

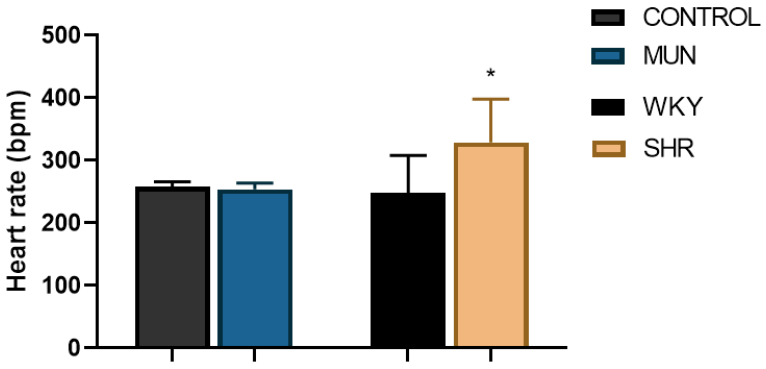

FIGURE 29| Heart rate of the 4 animal groups in study. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat**.** Values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

In awake MUN rats, it was confirmed that, at the age of 6 months, significantly higher SBP values (164.40±3.61 mmHg; *n*=6) were observed, compared to CONTROL animals (134.13±3.35 mmHg; *n*=6; *p*=0.003).

II. INFLUENCE OF FETAL UNDERNUTRITION ON VASCULAR MORPHOLOGY

Both morphology and collagen content were analysed in both models of hypertension. Histological sections of the mesenteric artery showed a reduced lumen diameter in MUN and SHR when compared to their respective control group (Figure 30A).

The quantitative analysis of the images demonstrated that:

i. The lumen area of MUN and SHR is significantly reduced when compared to their control (CONTROL and WKY, respectively) (Figure 30B).
ii. The media layer of MUN and SHR is significantly thicker when compared to their control (CONTROL and WKY, respectively) (Figure 30C).
iii. The adventitia layer of MUN is significantly thicker when compared to CONTROL (Figure 30D – left panel). The raise observed in SHR was not statistically different (Figure 30D – right panel).
iv. The ratio media/lumen and adventitia/lumen was significantly increased in MUN and SHR when compared to their control (CONTROL and WKY, respectively) (Figure 31).
v. A significant increase in collagen content is observed both in MUN and SHR when compared to their control (CONTROL and WKY, respectively) (Figure 32).

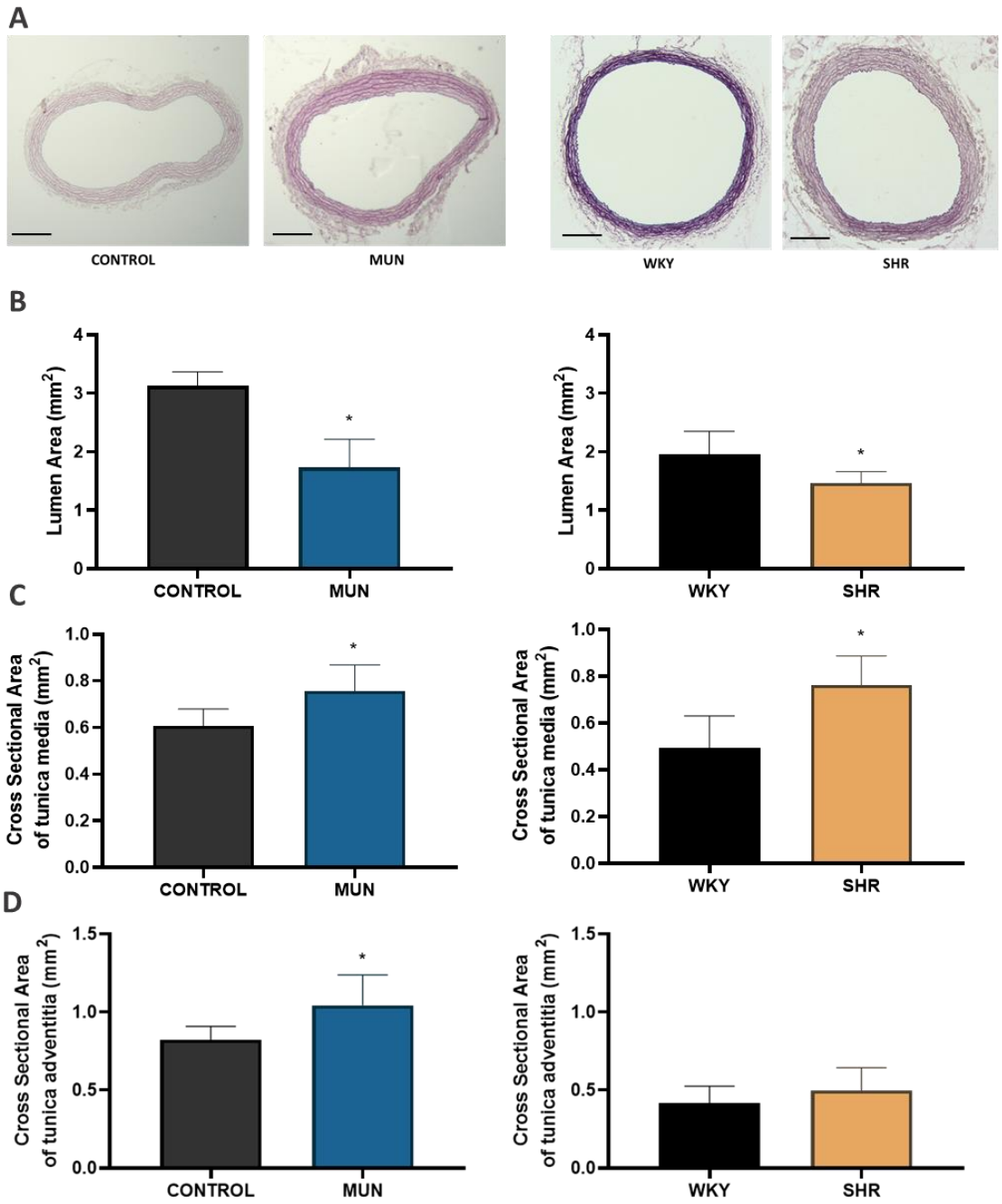

FIGURE 30| Representative images (A) and histomorphometric analysis (B, C and D) of mesenteric arteries from CONTROL and MUN (right panel) and WKY and SHR rats (left panel). Images were obtained from orcein stained arteries (scale bar = 500 µm). The graphics show the lumen area (B); cross sectional area of tunica media (C) and tunica adventitia (D). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

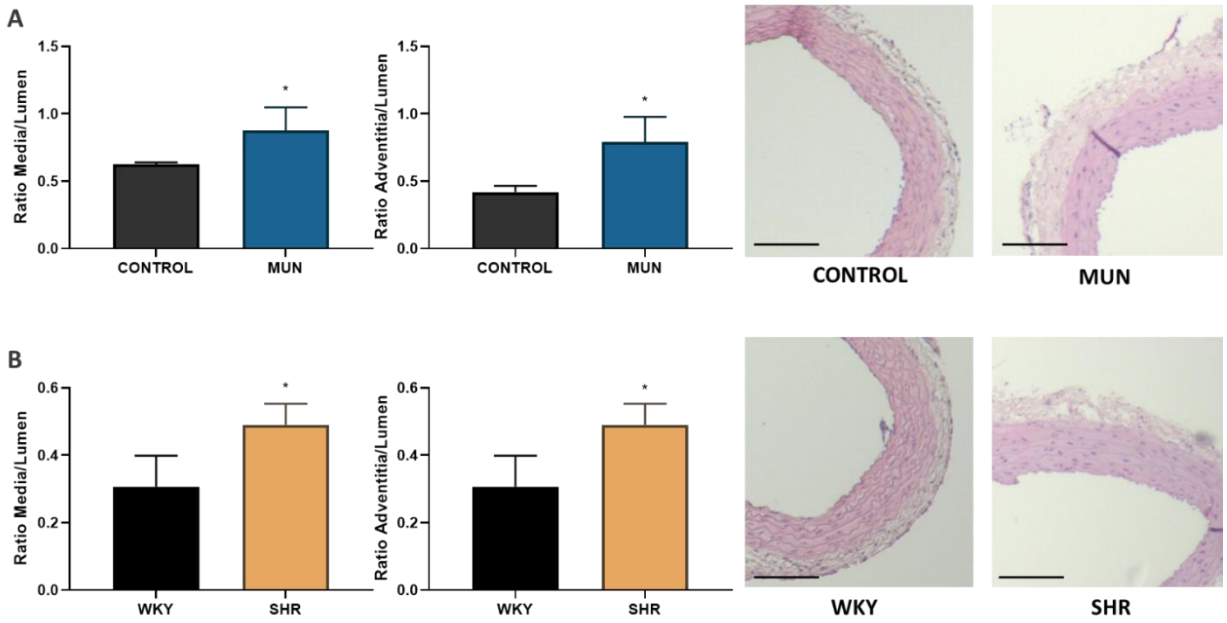

FIGURE 31| Histomorphometric analysis and representative images of mesenteric arteries from CONTROL and MUN (A) and WKY and SHR rats (B). The graphics show the ratio media/lumen (left) and the ratio adventitia/lumen (right). Images were obtained from hematoxylin-eosin stained arteries (scare bar = 300 µm). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

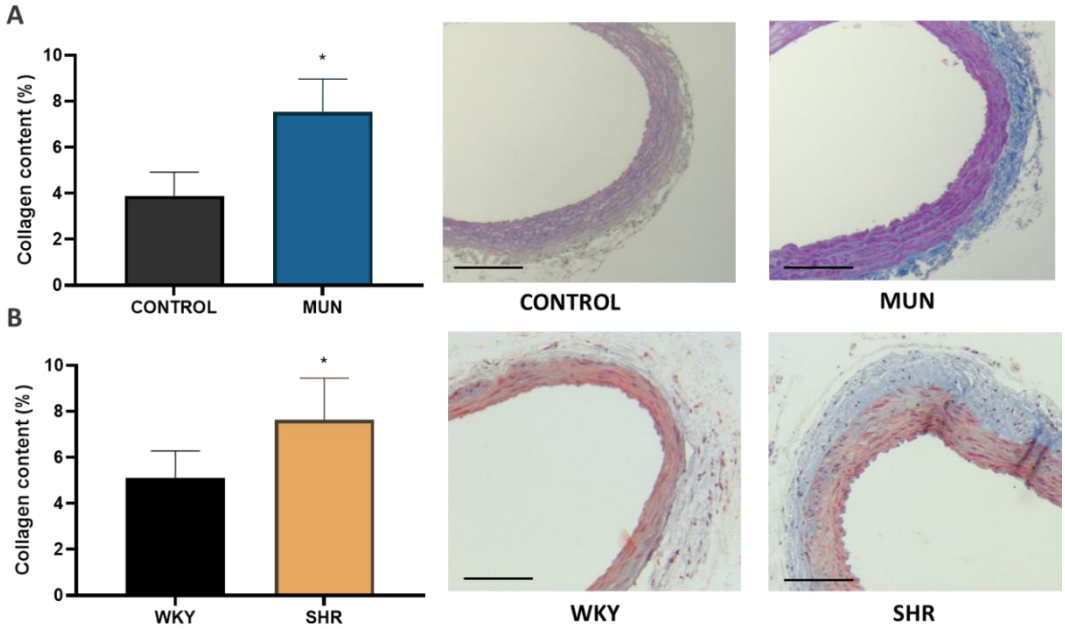

FIGURE 32| Perivascular collagen content and representative images of mesenteric arteries from CONTROL and MUN (A) and WKY and SHR rats (B). The graphics show the collagen content; results are expressed as percentage of the total artery area. Images were obtained from Masson's trichrome stained arteries (scare bar = 300 µm). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

III. INFLUENCE OF FETAL UNDERNUTRITION ON PERIVASCULAR SYMPATHETIC INNERVATION

i. SYMPATHETIC INNERVATION

Confocal microscopy studies were then carried out to evaluate the immunoreactivity for the sympathetic neuronal marker, TH. These studies were performed in the adventitial layer of mesenteric arteries which was identified from LSCM images by the shape and orientation of the nuclei and exhibiting scattered elastic fibres [228]. No significant immunoreactivity was observed when the primary antibodies were omitted (negative controls). Immunoreactivity for the sympathetic neuronal marker TH was evident in mesenteric arteries from all experimental groups (Figure 33, green marker). However, the pattern of TH immunoreactivity in MUN and in SHR was significantly larger than in CONTROL and WKY arteries, respectively (Figure 33).

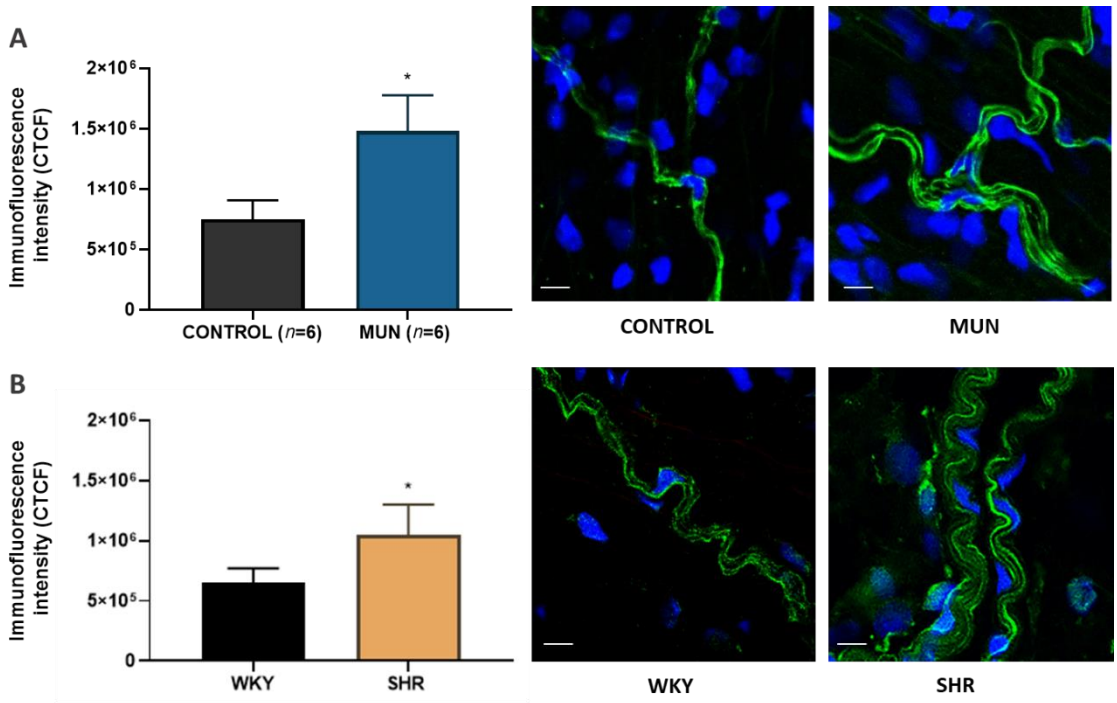

FIGURE 33| Quantitative analysis of LSCM images and representative images of the adventitia from mesenteric arteries from CONTROL and MUN (A) and WKY and SHR rats (B). Images shows the immunofluorescence reactivity to TH (green) with DAPI-stained nuclei (blue) (scare bar = 25 µm). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. In the graphics values are mean±s.e.m. from 6 rats of each group. Significant differences from the respective control rat: **p*<0.05.

ii. Sympathetic Neurotransmission

Electrical field stimulation (5 Hz, 1 ms, 100 pulses, 50 mA) significantly increased tritium outflow from all vessels studied of both MUN and CONTROL rats (Figure 34). The fractional rate of basal tritium outflow (b_1), electrically-evoked tritium overflow (S_1) of mesenteric arteries and veins and of caudal arteries are shown in Table 3. Basal outflow and electrically-evoked tritium overflow remained constant throughout the CONTROL experiments, with b_n/b_1 and S_n/S_1 values close to unity. Electrically-evoked tritium overflow (S_1) was higher in MUN compared to CONTROL vessels (Table 3).

Table 3| Basal and electrically evoked tritium overflow from MUN and CONTROL vessels.

		Basal Outflow (b_1) (fractional rate of outflow; min^{-1})	**Evoked Overflow (S_1)** (% of tissue tritium content)	**S_2/S_1**	***n***
Mesenteric Artery	**MUN**	0.093±0.007	0.348±0.029	1.076±0.009	5 *
	CONTROL	0.097±0.009	0.221±0.039	1.085±0.091	7
Mesenteric Vein	**MUN**	0.073±0.006	0.381±0.032	1.078±0.068	5 *
	CONTROL	0.088±0.006	0.265±0.021	0.992±0.057	7
Tail Artery	**MUN**	0.082±0.009	0.268±0.003	1.086±0.089	5 *
	CONTROL	0.072±0.007	0.163±0.045	1.001±0.098	7

Tissues were stimulated twice at 30-min intervals (S_1-S_2; 100 pulses, 5 Hz, 1 ms, 50 mA): b_1 refers to the 5-min period immediately before S_1. The electrically-evoked tritium overflow is expressed as a percentage of the tissue tritium content at the onset of stimulation. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m. and *n* denotes the number of tissue preparations used from 5 MUN and 5 CONTROL rats. Significant differences from the appropriate animal/tissue control: *p<0.05.

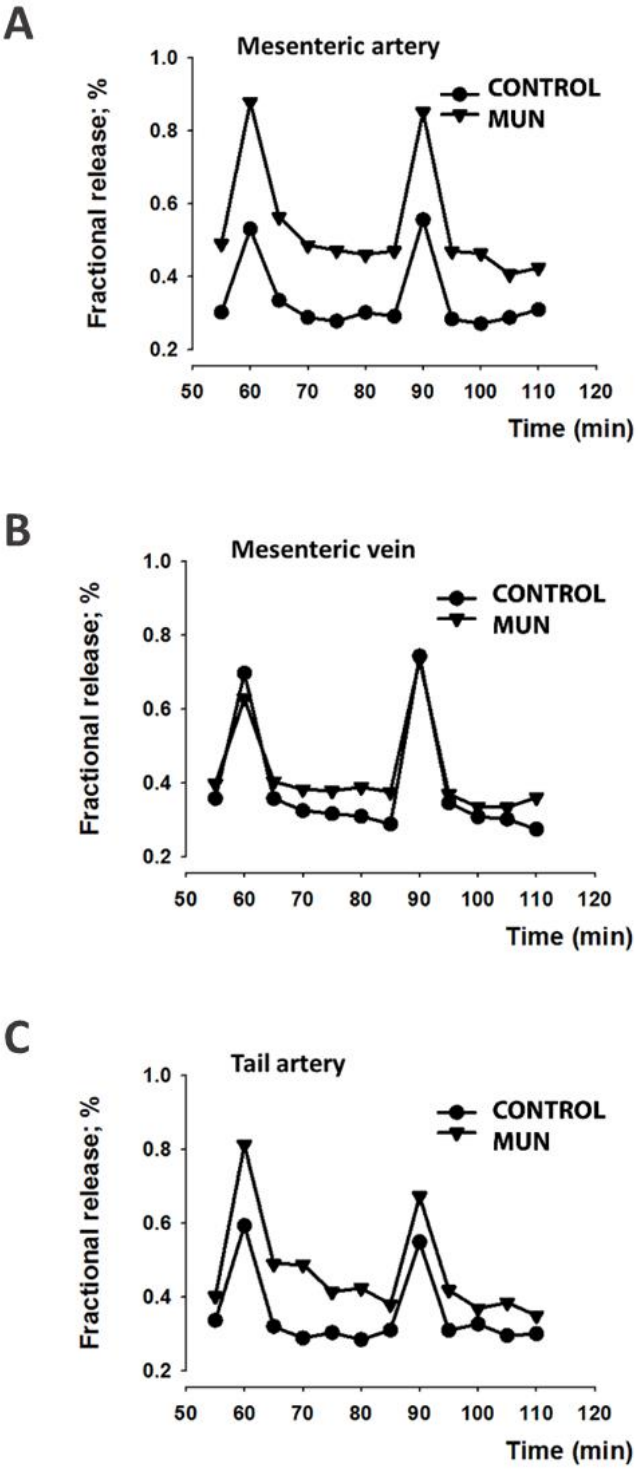

FIGURE 34| Representative examples of time course tritium outflow from mesenteric artery (A), mesenteric vein (B) and tail artery (C). Tritium outflow is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period. Each line represents the outflow of tritium from a single superfusion chamber. MUN (triangles), offspring exposed to maternal undernutrition during pregnancy; CONTROL (circles), offspring from mothers fed *ad libitum* during pregnancy.

Since results obtained were similar between the vessels in study, the mesenteric artery was used to make a comparison with SHR. A significant increase in NA released in the stimulation period was observed in SHR mesenteric arteries compared to WKY rats (Table 4 and Figure 35).

TABLE 4| Results of NA release experiments.

	Basal Outflow (b_1) (fractional rate of outflow; min^{-1})	**Evoked Overflow (S_1)** (% of tissue tritium content)	**S_2/S_1**
MUN	0.093±0.007	0.348±0.029*	1.076±0.009
CONTROL	0.097±0.009	0.221±0.039	1.085±0.091
SHR	0.071±0.003	0.358±0.05*	0.9873±0.037
WKY	0.070±0.008	0.229±0.037	0.9801±0.486

Tissues were stimulated twice at 30-min intervals (S_1-S_2; 100 pulses, 5 Hz, 1 ms, 50 mA): b_1 refers to the 5-min period immediately before S_1. The electrically-evoked tritium overflow is expressed as a percentage of the tissue tritium content at the onset of stimulation. Basal tritium outflow (b_1), electrically evoked tritium overflow (S_1) and S_2/S_1 ratios from the four animal groups in study. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m., from 6 rats of each group. Significant differences from the respective control rat: *p<0.05.

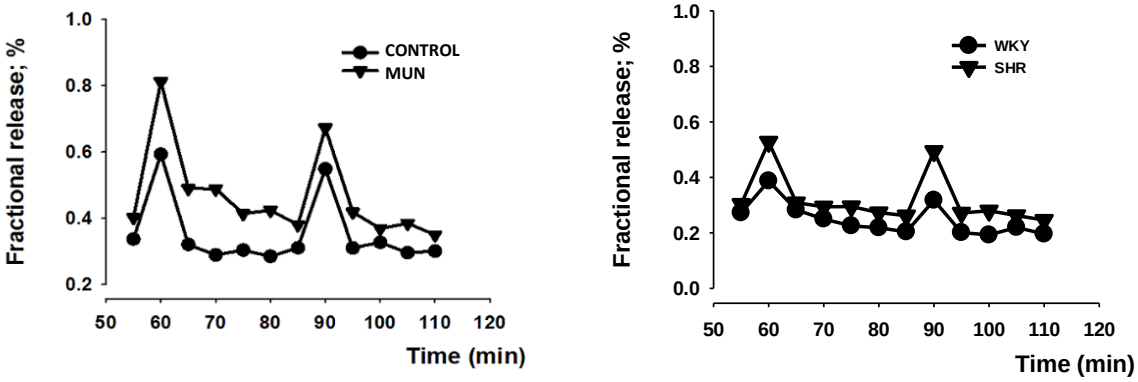

FIGURE 35 | Representative examples of time course tritium outflow from mesenteric artery of the 4 groups in study. On the right panel are the CONTROL (circles) and MUN (triangles) and on the left panel are the WKY (circles) and SHR (triangles). Each line represents the outflow of tritium from a single superfusion chamber. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

iii. [^{3}H]-NA UPTAKE

Since similar results were obtained between the vessels studied, the mesenteric artery was used to study the uptake of [^{3}H]-NA. In our experimental conditions, total tissue tritium content (per mg of tissue), after incubation with [^{3}H]-NA, was significantly higher in MUN than in CONTROL arteries (Figure 36).

[^{3}H]-NA uptake was significantly larger in MUN and SHR mesenteric arteries when compared to CONTROL and WKY, respectively (Figure 36).

Tissue content of NA was larger in CONTROL and MUN rats, compared to WKY and SHR.

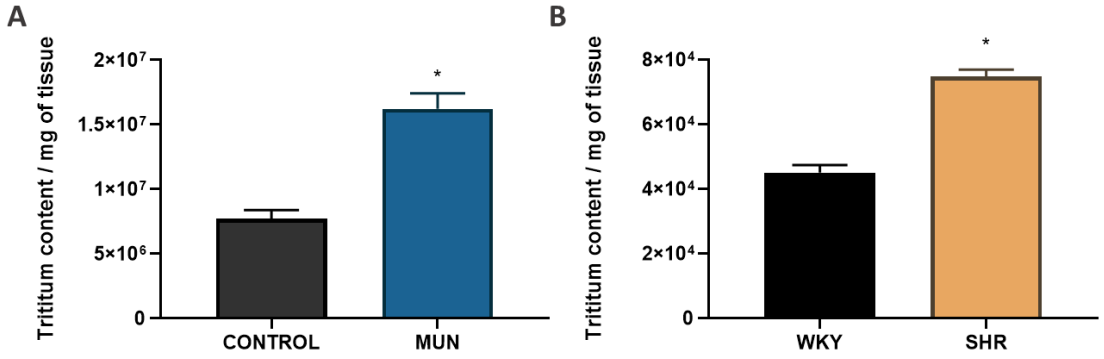

FIGURE 36| [^{3}H]-NA uptake in mesenteric artery of CONTROL and MUN (A) and WKY and SHR rats (B). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat. Values are mean±s.e.m., from 6 rats of each group. Significant differences from the respective control rat: *p<0.05.

IV. INFLUENCE OF FETAL UNDERNUTRITION ON RENIN-ANGIOTENSIN SYSTEM

i. ROLE OF ANG II IN VASCULAR SYMPATHETIC NEUROTRANSMISSION

Ang II (30 nmol/L; a non-selective agonist of angiotensin receptors) facilitated electrically-evoked tritium overflow in all the vessels studied (mesenteric artery, mesenteric vein and tail artery) from both experimental groups, as shown by the ratio (S_2/S_1) in the absence of the drug. No statistical differences were observed between different vessels or between different experimental groups (Figure 37).

Endogenous Ang II-mediated effects on vascular sympathetic neurotransmission were evaluated by blocking the AT_1 receptor with Losartan, a selective AT_1 receptor antagonist. In the presence of Losartan (100 nmol/L), an inhibition of electrically-evoked tritium overflow was observed in all the MUN vessels studied (Figure 37). Losartan was unable to modify tritium overflow in vessels from CONTROL rats.

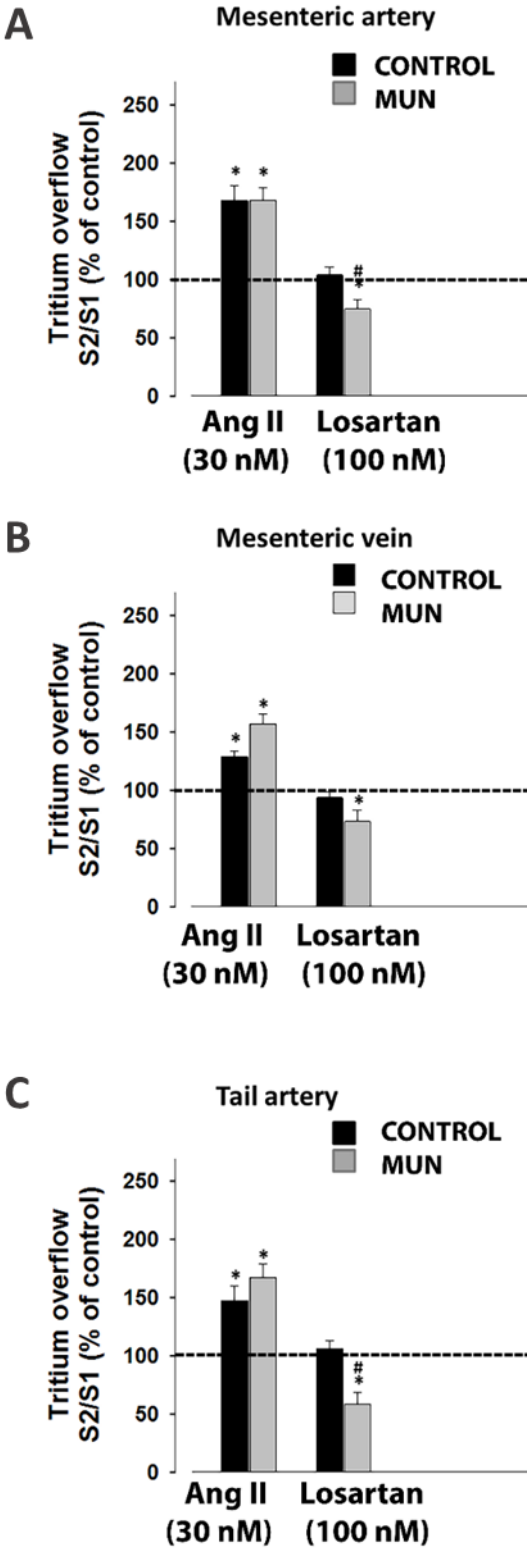

FIGURE 37| Effects of Ang II and Losartan on the electrically-evoked tritium overflow in mesenteric artery (A), mesenteric vein (B) and tail artery (C) from CONTROL and MUN rats. Ordinates: S_2/S_1 values obtained in individual tissue preparations, expressed as a percentage of the appropriate S_2/S_1 control value. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m. from 5 to 16 tissue preparations from 6 rats of each group. Significant differences from CONTROL rats: **p*< 0.05; from the control vessels: #*p*< 0.05.

ii. Plasmatic Angiotensin II

No significant differences in Ang II were detected between MUN and Control rats (Figure 38).

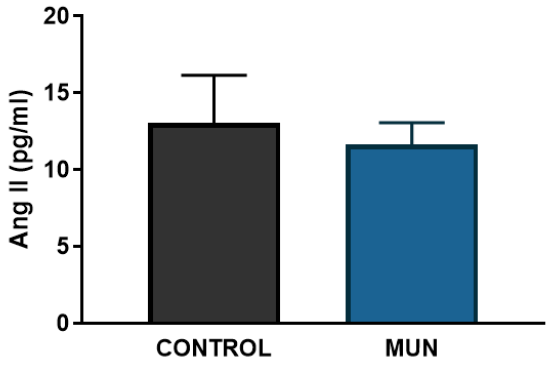

Figure 38| Plasmatic concentration of Ang II in CONTROL and MUN rats. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m., from 8 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

iii. Expression of RAS enzymes in the mesenteric artery

a. Renin

Statistical differences were not detect in renin expression in the mesenteric artery between MUN and CONTROL rats (Figure 39).

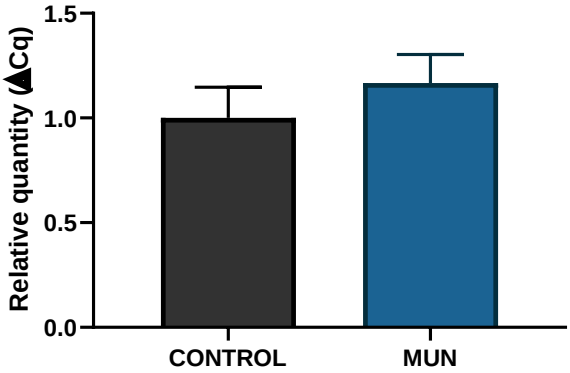

Figure 39| Expression of renin in mesenteric arteries from MUN and CONTROL rats. RT-PCR analysis of transcripts for renin. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m., from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

b. *Angiotensin Converting Enzyme*

Concerning ACE, the enzyme that converts Ang I into Ang II, it was found a significant higher expression in mesenteric artery from MUN rats when compared to CONTROL rats (Figure 40).

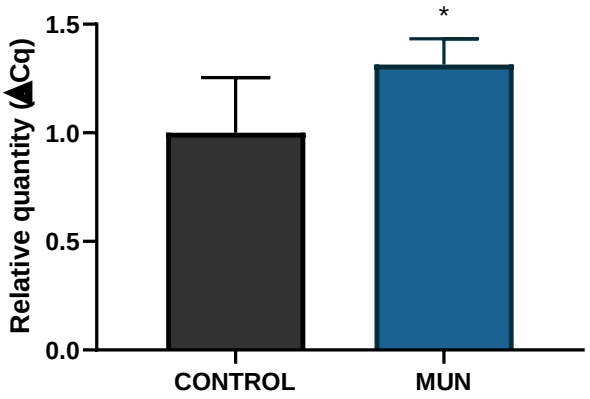

FIGURE 40| Expression of ACE in mesenteric arteries from MUN and CONTROL. RT-PCR analysis of transcripts for ACE. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean ± s.e.m., from 6 rats of each group. Significant differences from CONTROL rats: *p<0.05.

c. *Angiotensin Converting Enzyme 2*

Regarding ACE 2, the enzyme that converts Ang II in Ang 1-7, it was found a significant higher expression in MUN rats when compared to CONTROL rats (Figure 41).

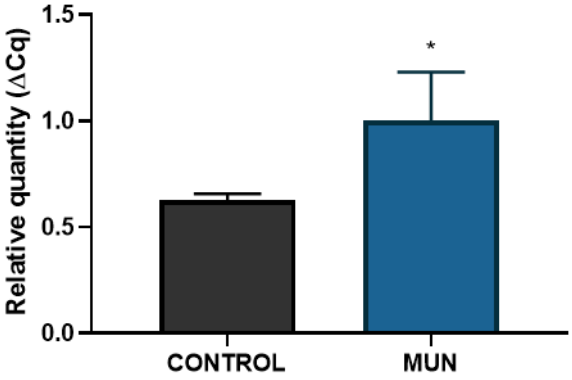

FIGURE 41| Expression of ACE 2 receptor in mesenteric arteries from MUN and CONTROL. RT-PCR analysis of transcripts for ACE2. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m, from 6 rats of each group. Significant differences from CONTROL rats: *p<0.05.

iv. Expression of Angiotensin Receptors in the Mesenteric Artery

a. AT_1 Receptors

RT-PCR data revealed a higher expression of AT_1 receptor in the mesenteric artery of MUN when compared to CONTROL rats (Figure 42).

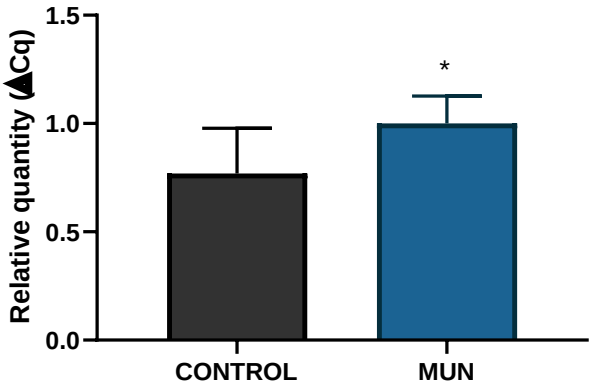

Figure 42| Expression of AT_1 receptor in mesenteric arteries from MUN and CONTROL rats. RT-PCR analysis of transcripts for AT_1 receptor. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m from 6 rats of each group. Significant differences from CONTROL rats: *p<0.05.

In addition, LSCM revealed the presence of AT_1 receptor immunoreactivity in both CONTROL and MUN mesenteric arteries (Figure 43A, red images). However, in MUN arteries the quantitative analysis evidenced that the relative amount of AT_1 receptor immunoreactivity was much higher (up to 80% higher) than that exhibited by CONTROL mesenteric arteries (Figure 43B).

The localization pattern of AT_1 receptors in sympathetic nerves, stained with TH, were analysed. The immunoreactivity for AT_1 receptors showed substantial overlay with TH marker suggesting that these receptors might be localized on the same structure, the postganglionic sympathetic nerves (Figure 43A, overlay images). Nonetheless, immunoreactivity for AT_1 receptors in non-neuronal cells could also be observed.

Quantitative analysis of LSCM images revealed considerable differences in the relative amount of TH, AT_1 receptor and AT_1-TH overlay between the two experimental animal

groups: values obtained from MUN animals almost doubled comparatively to CONTROL rats (Figure 43B).

Relative mean ratio of AT_1 receptor and TH overlay, with respect to the total TH immunoreactivity, was slightly higher in MUN comparatively to CONTROL mesenteric arteries: 25% of sympathetic neurons exhibit AT_1 receptor immunoreactivity in CONTROL versus 35% observed in MUN vessels (Figure 43C). Also, the relative mean ratio of AT_1 receptor and TH overlay, regarding the total AT_1 immunoreactivity revealed the presence of AT_1 receptors in other cells than sympathetic neurons: 70% in CONTROL and 60% in MUN mesenteric arteries (Figure 43D).

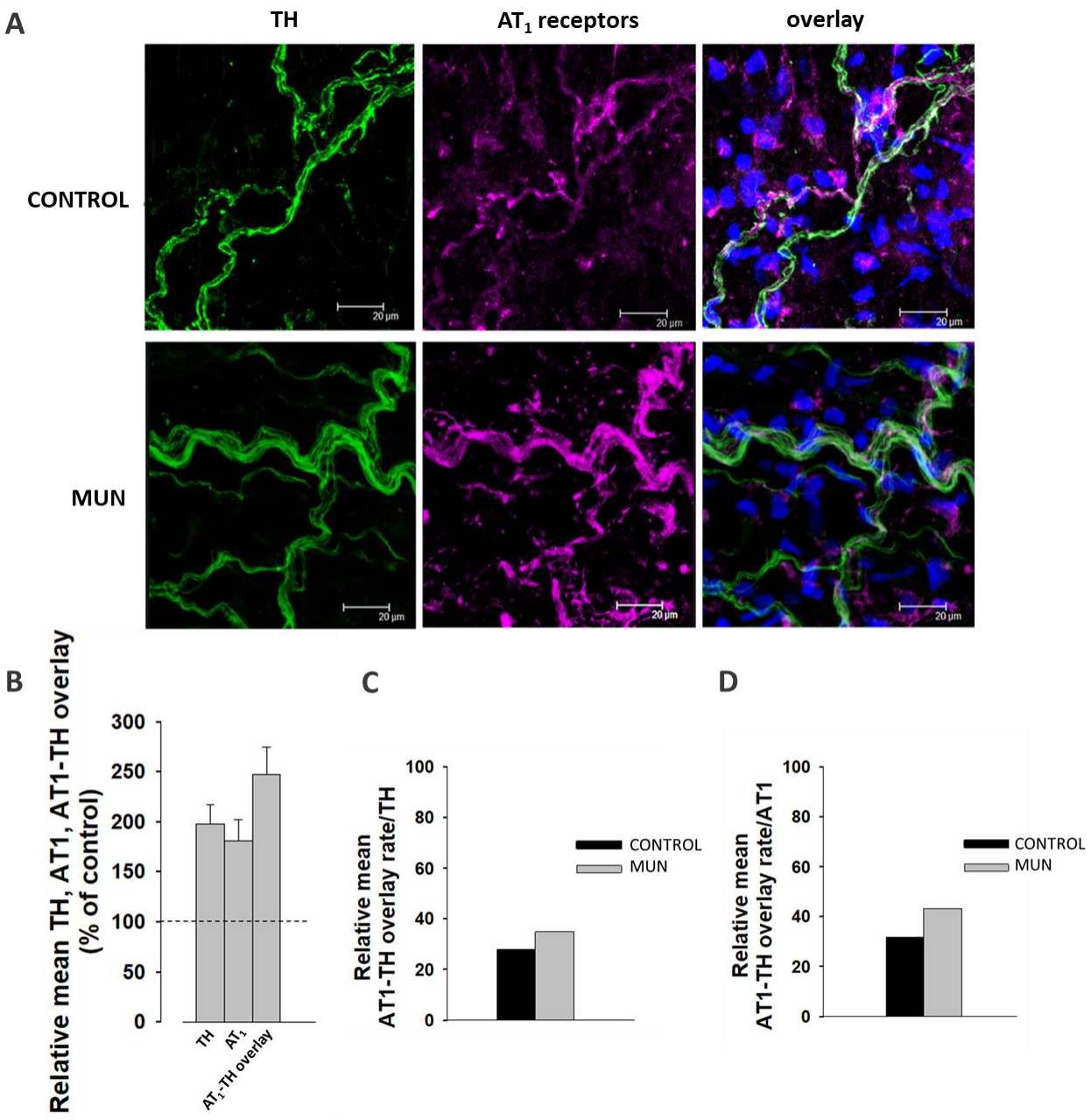

FIGURE 43| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries (A) exhibiting TH (green), AT_1 receptor (red), and overlay of AT_1 receptor-TH immunoreactivities, nuclei (blue); Relative means of TH, AT_1 and AT_1-TH overlay expressed as percentage of CONTROL values (B); Mean percentage of overlay ratio with TH (C) and mean percentage of overlay ratio with AT_1 receptors (D). Images are reconstructions from 9–28 serial optical sections analysed using PAQI software. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m. from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

b. AT_2 Receptors

RT-PCR data revealed a lower expression of AT_2 receptors in the mesenteric artery of MUN, when compared to CONTROL rats (Figure 44).

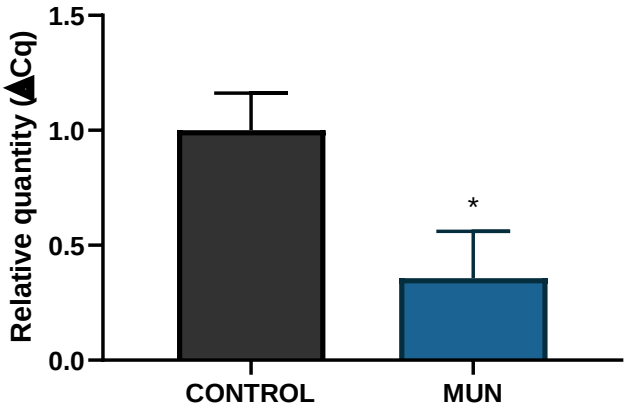

FIGURE 44| Expression of AT_2 receptor in mesenteric arteries from MUN and CONTROL rats. RT-PCR analysis of transcripts for AT_2 receptor. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m from 6 rats of each group. Significant differences from CONTROL rats: *p<0.05.

LSCM also revealed the presence of AT_2 immunoreactivity in both CONTROL and MUN mesenteric arteries (Figure 45, red images). Quantitative analysis evidenced that the relative amount of AT_2 immunoreactivity present in MUN arteries was reduced (up to 40% lesser) than that exhibited by CONTROL vessels (Figure 45B).

It was observed an immunoreactivity for AT_2 receptors in non-neuronal cells (Figure 45A).

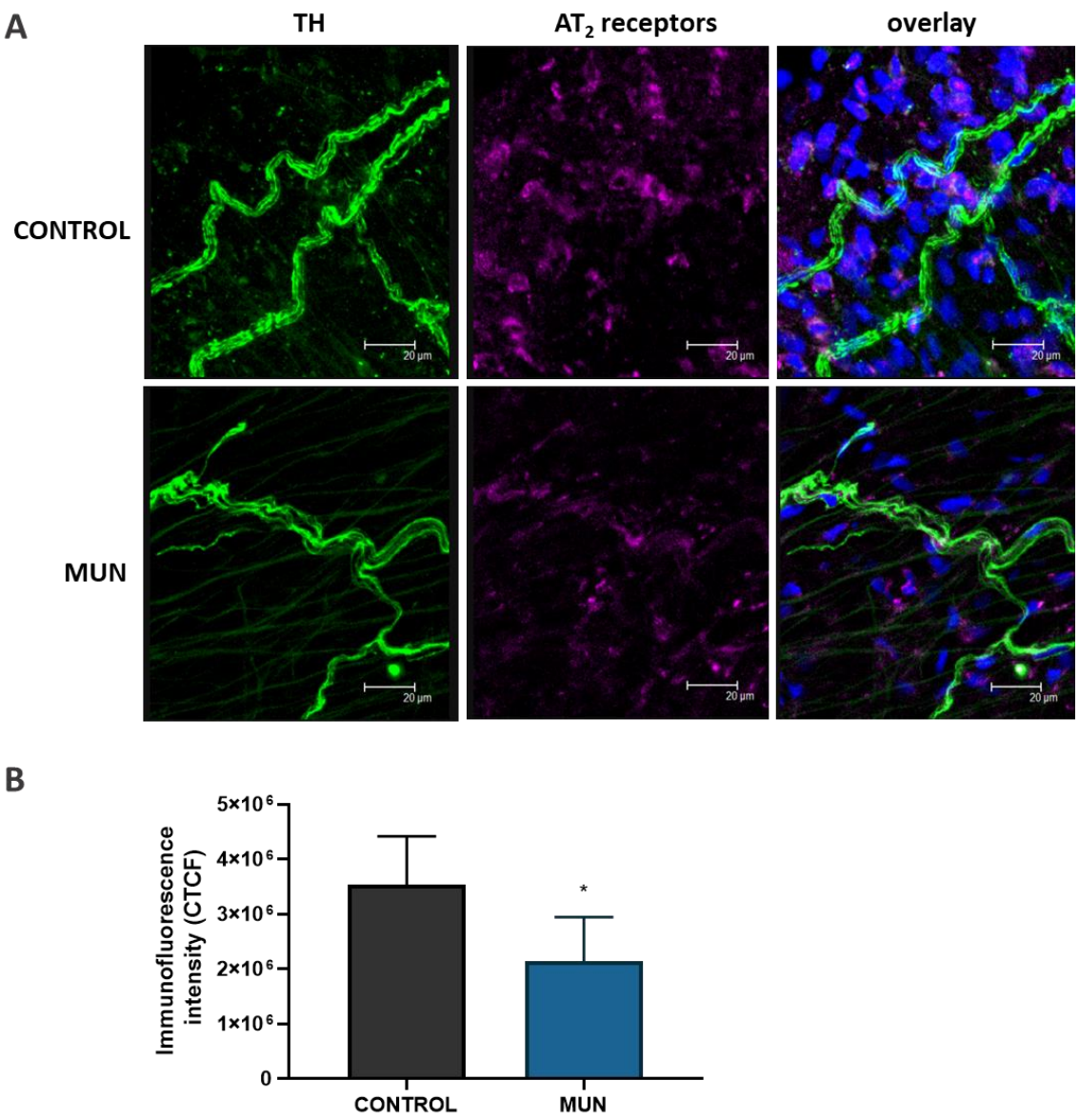

FIGURE 45| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries (A) exhibiting TH (green), AT_2 receptor (red), and overlay of AT_2-TH immunoreactivities, nuclei (blue) and quantitative analysis of LSCM images from mesenteric arteries (CONTROL and MUN) staining with AT_2 antibodies (B). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m. from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

c. Mas Receptors

RT-PCR data revealed a lower expression of Mas receptors in the mesenteric artery of MUN, when compared to CONTROL rats (Figure 46).

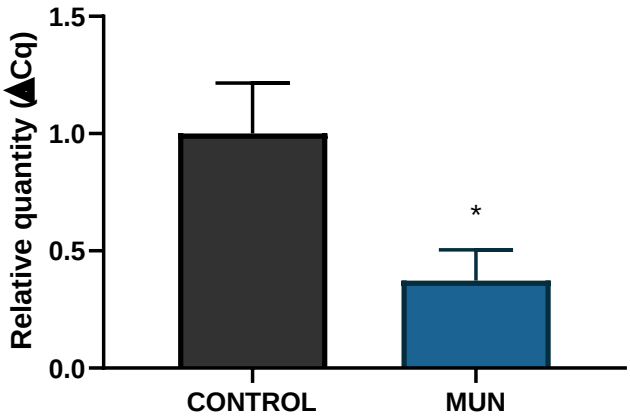

FIGURE 46| Expression of Mas receptor in mesenteric arteries from MUN and CONTROL rats. RT-PCR analysis of transcripts for Mas receptor. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m from 6 rats of each group. Significant differences from CONTROL rats: *p<0.05.

This quantification was in line with our confocal study for this type of receptor, a significant lower immunoreactivity for Mas receptors was found in MUN rats when compared to CONTROL rats (Figure 47).

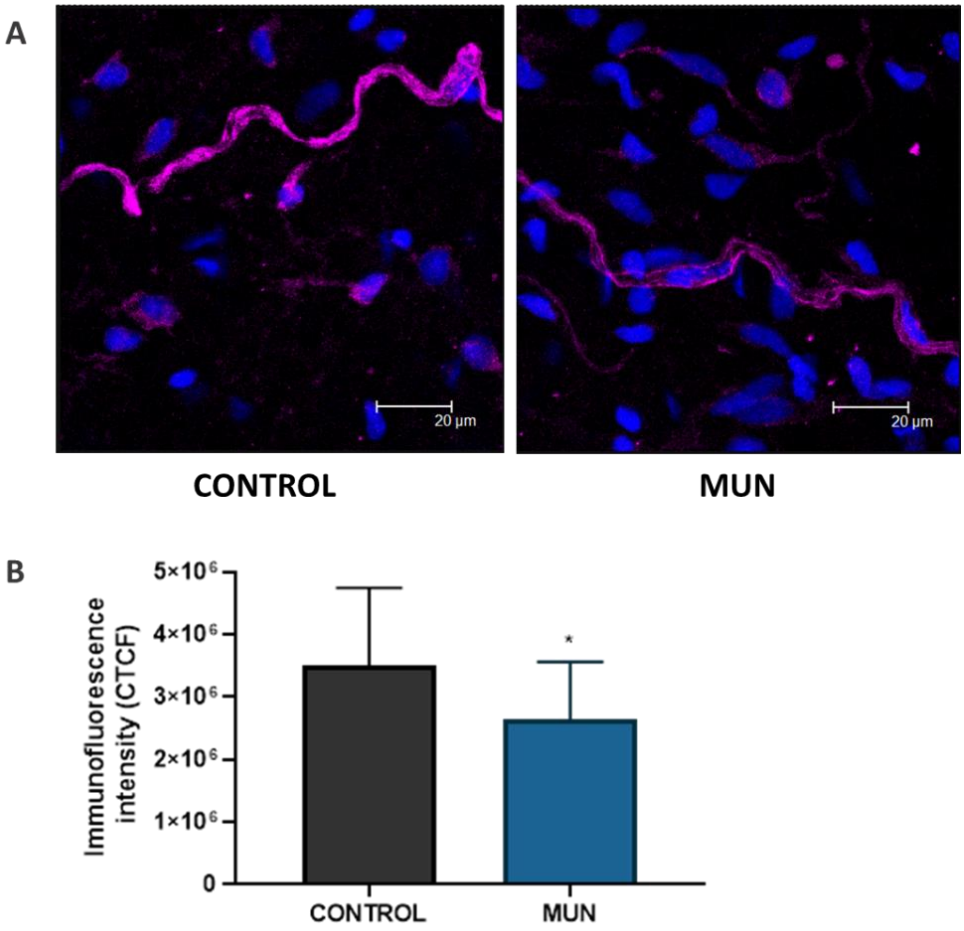

FIGURE 47| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries exhibiting Mas receptors (red) and nuclei (blue) (A) and quantitative analysis of LSCM images (B). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are expressed as mean±s.e.m. from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

d. MrgD Receptors

RT-PCR data revealed a significant lower expression of MrgD receptors in the mesenteric artery of MUN, when compared to CONTROL rats (Figure 48).

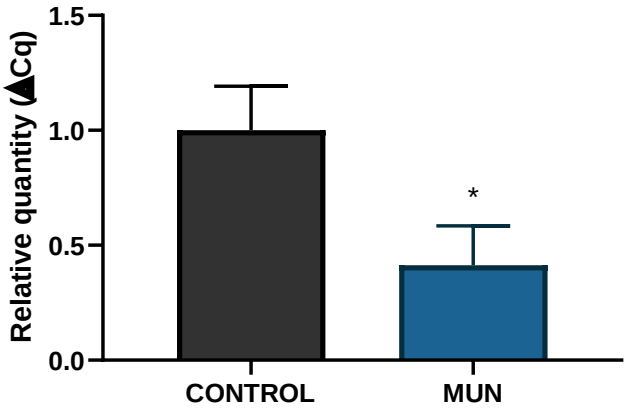

FIGURE 48| Expression of MrgD receptor in mesenteric arteries from MUN and CONTROL rats. RT-PCR analysis of transcripts for MrgD receptor. MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Data are normalized to the 28s gene and fold changes between MUN and CONTROL are expressed as mean±s.e.m from 6 rats of each group. Significant differences from CONTROL rats:*p<0.05.

A lower expression of MgD receptor in MUN rats was also confirmed by the confocal study and respective quantitative analysis (Figure 49).

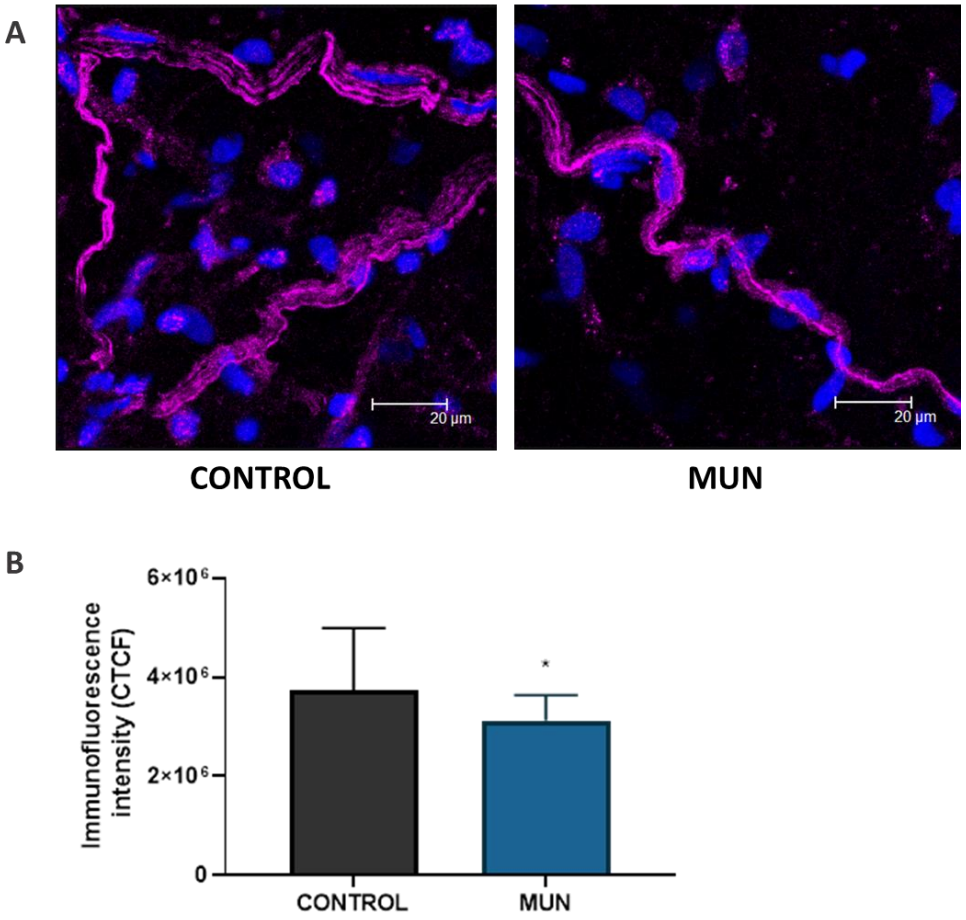

FIGURE 49| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries exhibiting MrgD receptors (red) and nuclei (blue) (A) and quantitative analysis of LSCM images (B). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

v. Localization of RAS Receptors

During confocal study of RAS receptors, it was noticed the presence of receptors in other cells besides sympathetic neurons. The presence of AT_1 receptors on Schwann cells was investigated. To localize Schwann cells, anti-GFAP-immunoreactivity was used.

Data from LSCM images evidenced AT_1 receptors and GFAP overlaid immunoreactivities (Figure 50), confirming the presence AT_1 receptors on Schwann cells. In addition, other adventitial cells also exhibited immunoreactivity against AT_1 receptors.

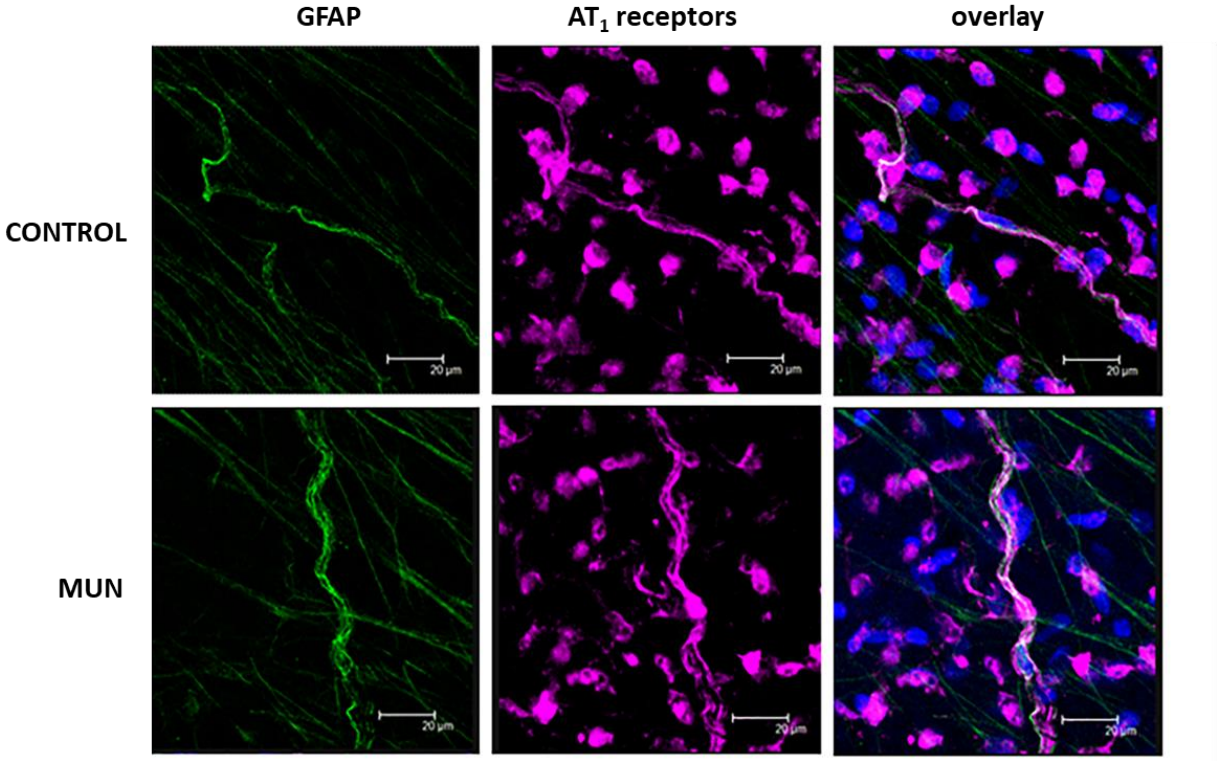

Figure 50| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries exhibiting GFAP (green), AT_1 receptors (red), and overlay of AT_1 receptors-GFAP immunoreactivities, nuclei (blue). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy.

The possible location of RAS receptors within the nucleus was also investigated. It was found that AT_1 and AT_2 receptors, Mas and MrgD exhibited immunofluorescence inside the nucleus of adventitial cells (Figure 51, grey arrows).

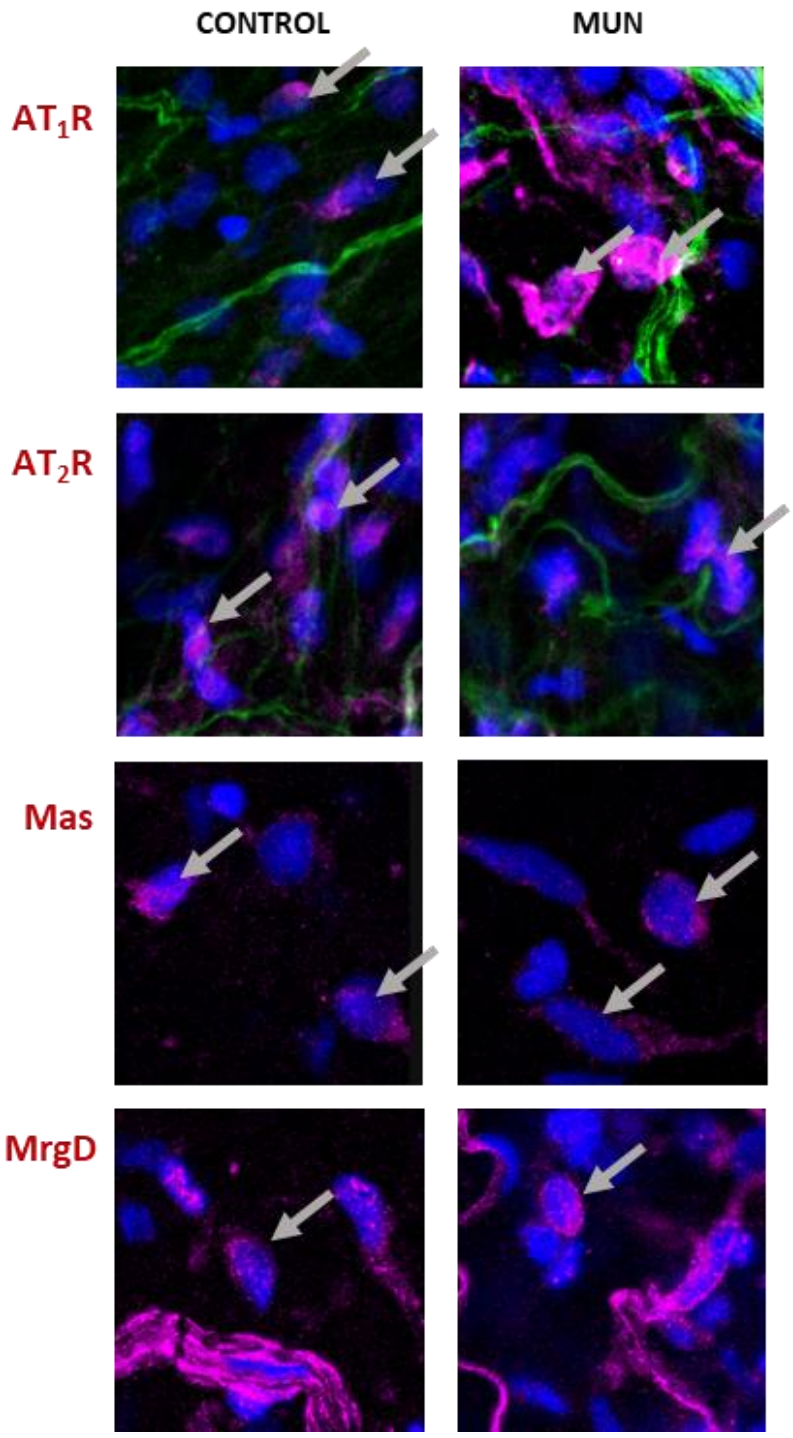

FIGURE 51| Laser scanning confocal microscopy representative images of MUN and CONTROL mesenteric arteries exhibiting RAS receptors (red) and nuclei (blue). MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy.

V. IMPACT OF FETAL UNDERNUTRITION IN $\bullet O_2^-$ PRODUCTION

A higher immunoreactivity for p22phox, a subunit of NADPH oxidase, was observed in MUN rats, when compared to CONTROL (Figure 52A). This observation was in line with the quantitative analysis of the images collected, where a significant higher p22phox immunoreactivity was found in MUN rats (Figure 52B).

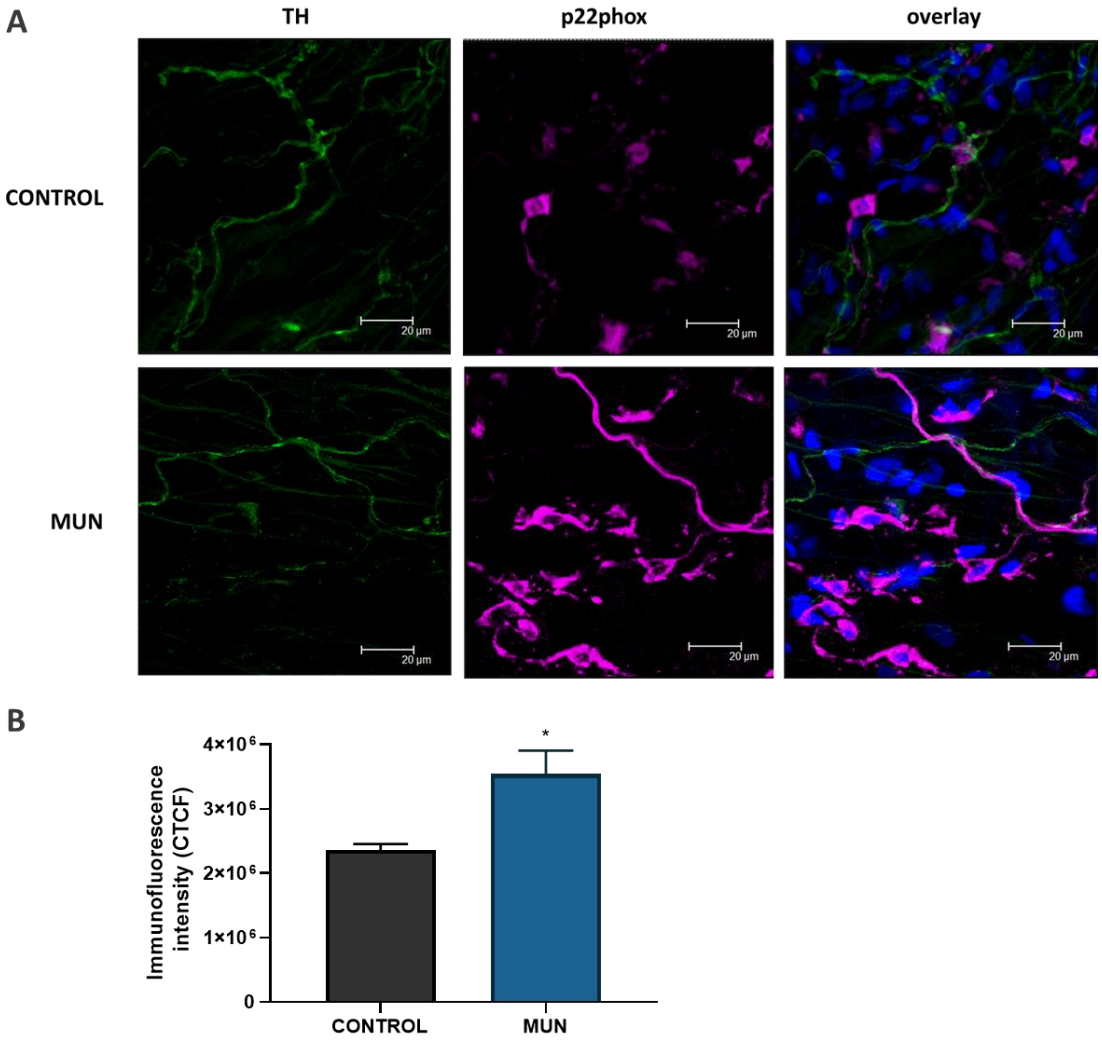

FIGURE 52| Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries (A) exhibiting TH (green), p22phox (red), and overlay of p22phox-TH immunoreactivities, nuclei (blue); **and quantitative analysis of LSCM images (B).** MUN, offspring exposed to maternal undernutrition during pregnancy; CONTROL, offspring from mothers fed *ad libitum* during pregnancy. Values are mean±s.e.m. from 6 rats of each group. Significant differences from CONTROL rats: **p*<0.05.

Discussion

At present, CVDs continue to be the leading cause of death and disability in the world, responsible for almost one third of global deaths, being essential hypertension one of the most important risk factors. Epidemiological studies have consistently found an association between exposure to fetal stress, LBW and increased risk in adulthood to develop hypertension and CVDs, a phenomenon known as fetal programming. Additionally, experimental and clinical studies have demonstrated that sustained maternal nutrient restriction, a well-known fetal stress factor, activate adaptive cardiac and vascular changes that might explain this association. However, the mechanisms underlying the observed cardiovascular alterations are not fully understood, and more research is needed to identify strategies to prevent and/or treat FPH, aiming to reduce the socioeconomic impact of CVDs.

Over the past years, some studies have addressed the activation of SNS and RAS as possible mechanisms implicated in FPH. Increasing clinical evidence indicates a key role for sympatho-activation in the development of CVDs, highlighting the need gain insight on how the SNS interfaces with the cardiovascular system and how this interaction may result in increased morbidity or mortality. The RAS is also an important system implicated in hypertension, and a target in the current treatment mainly directed to the renin/ACE/Ang II/AT_1 receptor axis. Moreover, the ACE2/Ang 1-7/Mas and MrgD axis is a possible future therapeutic target. This work aimed to develop new knowledge about the role of SNS and RAS in FPH and to study the possible interplay between these two systems. Another aim of this work was to compare the alterations induced by fetal stress (undernutrition) and those found in SHR, a well-known model of essential hypertension, to determine if hypertension of fetal origin shares similitudes with essential hypertension.

To address this, an animal model of FPH based on maternal undernutrition during pregnancy (MUN) was used, which is known to produce offspring with LBW that, later in life, develop hypertension [184, 220, 230]. This model was chosen, instead of the one based on maternal low protein diet, since it better resembles suboptimal nutrition to the fetus in humans, induced either by maternal malnutrition or by placental insufficiency. In both situations, there is a global reduction of nutrient access to the fetus, rather than just a reduction in protein accessibility.

In order to facilitate the discussion, at the beginning of each section a scheme that summarizes the most relevant results is included.

i. Hemodynamic alterations induced by fetal undernutrition. Comparison with SHR model.

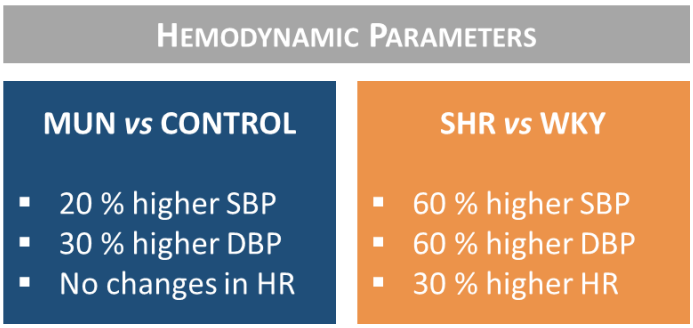

Figure 53| Hemodynamic parameters alterations in MUN (blue) and SHR (orange).

BP was assessed by two methods, direct measurement in anesthetized rats through the femoral artery, and in awake animals with plethysmography. It was demonstrated that at the age of 6 months MUN male rats had reached a hypertensive state, since they presented a significant higher SBP and DBP than CONTROL rats. These findings confirm previous results with the same model of fetal stress induced by global nutrient restriction during intrauterine life [108, 204, 230-232].

SHR also exhibited higher values of SBP and DBP than the control WKY, as shown in many studies with this rat strain [233-235]. Comparing both animal models of hypertension, the increase in SBP and DBP was higher in SHR, than in MUN rats.

No differences were found in HR of anesthetized MUN and CONTROL rats, similar to the findings in other studies using maternal low protein [182, 236], or low micronutrients -such as zinc [237]- or vitamin B12 [238] diets during pregnancy. However, in SHR, HR was elevated. This tachycardia may reflect an alteration in baroreceptors regulation, which has been demonstrated to be defective in SHR rats [239]. It is possible that in SHR the increased heart rate may contribute to the higher blood pressure compared to MUN rats.

To anesthetize the rats, medetomidine was used, a drug which has an agonist effect on α_2-adrenoceptors. This anaesthetic has been reported to reduce NA outflow within the central nervous system, dampening the central sympathetic tone and resulting in bradycardia [240, 241]. Therefore, to test the possible influence of anaesthesia on BP, measurement with the tail-cuff method, in awake rats, was performed. Similar results were obtained, confirming the development of hypertension in MUN rats.

Fetal undernutrition induces in male rat offspring high BP, with no alterations in HR, in adulthood.

Fetal undernutrition induces a milder form of hypertension than in a genetic model of essential hypertension.

ii. Vascular remodelling induced by fetal undernutrition. Comparison with SHR model.

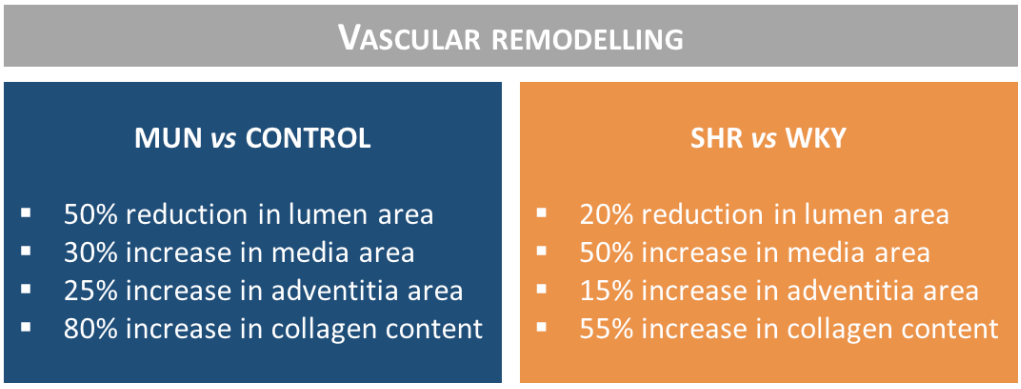

FIGURE 54| Vascular morphological alterations in MUN (blue) and SHR (orange).

In a healthy artery, sustained changes in the extravascular environment, local signaling molecules, or hemodynamic modifications, initiate structural adaptations within the different cell types and layers of the vessel wall. Once the initial stimulus is restored, the vascular wall returns to the original state. Nevertheless, in disease states, these changes do not return to baseline levels, but instead initiate pathological vascular alterations observed in CVDs, in a process defined as "vascular remodelling" [242].

Vascular remodelling is a heterogeneous process and differs depending on the vessel type and specific disease state or progression. In the majority of studies of hypertensive remodelling, resistance vessels have been used, since they contribute to peripheral resistance and are usually affected in hypertension [30, 243]. In this study the superior mesenteric artery was chosen, since it is an important vessel which contributes to BP maintenance [211, 244].

The mesenteric artery of MUN rats exhibit a lower lumen area and an increased media and adventitia layers compared to the CONTROL. These geometrical changes are classified as inward hypertrophic vascular remodelling. This type of remodelling is characteristic of hypertension, and can contribute to an increased total peripheral vascular resistance [30]. In mesenteric resistance arteries from rats exposed to undernutrition in utero, the same type of remodelling has also been found [245], while the aorta, a conduit artery, presented outward hypertrophic remodelling [232, 245]. These data indicate that different vessels exposed to the same stress factor undergo different types of structural adaptations.

The hypertrophy observed in the media layer in MUN rats may be due to cellular hypertrophy caused by VSMC proliferation or growth. Previous studies demonstrated that offspring exposed to maternal undernutrition have reduced angiogenesis and consequently, a decreased number of mesenteric microvessels at birth, which may induce a flow stress and elicit a compensatory increase in VSMC [246]. This has also been observed in the aorta from this rat model of undernutrition, which exhibit hypotrophy at birth followed by hypertrophy in the perinatal period which is maintained in adult age [232]. Excess GC may also be implicated in the abnormal growth and differentiation of VSMC, since they have been shown to be elevated in the maternal circulation of food-restricted rats [245]. Besides these factors, Ang II and NA are well known trophic factors and an elevation can also favour the hypertrophy of VSMC [243, 247]. Sympathetic hyper-innervation was shown to be related to media hypertrophy in jejunal arteries of SHR, since an increase in the number of nerve fibres was present before the development of hypertension, or increase in thickness of the arterial media [248]. This suggests that increased SNA possible plays a causal role in the development of hypertension, through vascular remodelling.

It has been demonstrated that increased VSMC could influence the expression of vascular extracellular matrix components, such as collagen. In animal models of FPH increased collagen has been observed in the aorta [246]. Similarly, in the mesenteric artery of MUN rats collagen content was also increased. This is an important finding in MUN rats since collagen is the extracellular component that may alter the passive pressure/diameter relation of arteries at higher pressures and thus, induce a progressive stiffening of the vascular wall [15]

In addition to VSMC, collagen can be also generated by fibroblasts in the adventitial layer; it has been proposed that phenotypic changes of these cells into myofibroblasts, play a major role in arterial remodelling and matrix deposition [249]. The adventitia has not been well explored in the context of FPH, and to our best knowledge, this is the first study demonstrating that mesenteric arteries of MUN rats have significantly increased adventitia area and collagen content. Furthermore, the presence of an increased media/lumen ratio and adventitia/lumen ratio in MUN rats was also demonstrated. Since wall/lumen is associated with higher risk of adverse cardiovascular events [28, 29], the remodelling process in MUN rats does not seem to be adaptive and is likely to contribute to cardiovascular damage.

The alterations in vascular morphology of mesenteric arteries of MUN and CONTROL rats were compared with the results obtained for SHR, a well-known model of hypertension with structural alterations in both conduit [250] and resistance vessels [251-253]. This study demonstrate that in the mesenteric artery of SHR an inward hypertrophic vascular remodelling was observed. In mesenteric resistance vessels from SHR inward eutrophic remodelling occurs while in models of secondary hypertension hypertrophy has been observed [31]. However, fibrosis and excessive extracellular matrix deposition seems to be a common denominator in different types of hypertension [18, 19]. Our study demonstrates that the remodelling process is relatively similar in MUN and SHR rats (inward hypertrophic), with a larger contribution of the adventitial layer in the MUN model.

Fetal undernutrition induces inward hypertrophic remodelling in the mesenteric artery, with the contribution of both the media and the adventitia layers, and the presence of vascular fibrosis.

Fetal undernutrition and essential hypertension share similar characteristics of vascular remodelling in the mesenteric artery.

iii. Alterations in sympathetic neurotransmission induced by fetal undernutrition. Comparison with SHR.

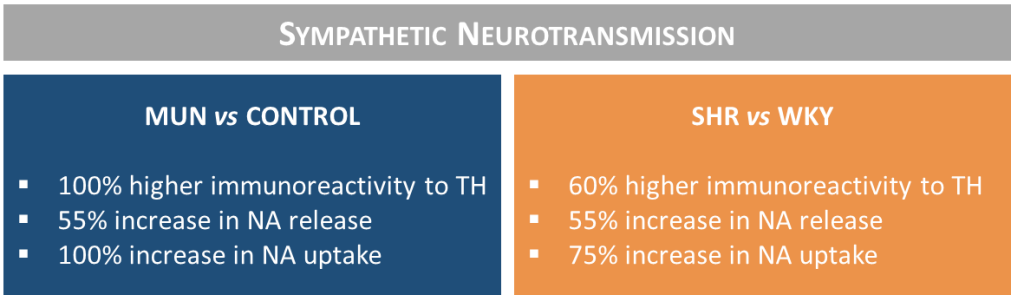

Figure 55| Vascular sympathetic neurotransmission alterations in MUN (blue) and SHR (orange).

The hypothesis that essential hypertension may include, in its multifactorial etiology, an abnormality in the autonomic modulation of BP homeostasis has been investigated for a long time. This process involves a sympathetic activation coupled with a parasympathetic inhibition, called "neurogenic hypothesis of hypertension" [71]. Indeed, the SNS activity can elevate BP by: augmenting the force and/or rate of cardiac contraction; decreasing the diameter of resistance arteries; and reducing sodium and water excretion by the kidneys [254].

In the present work, confocal microscopy was used to analyse sympathetic innervation with an antibody against TH. This is a technique that allow studying the entire adventitial layer without the need to dissect it. LSCM data revealed the presence of nerve fibres positive for TH both in CONTROL and MUN. We observed that sympathetic nerves spread through the adventitia reaching the medial layer. There was, however, a larger thickness of sympathetic nerve fibres in MUN mesenteric arteries. The higher immunoreactivity for TH observed in MUN rats indicates a sympathetic hyper-

innervation induced by exposure to undernutrition during fetal life. This is in accordance with the findings in other FPH animal model, such as induced by prenatal hypoxia, which demonstrated a sympathetic hyper-innervation in tibial arteries [173].

In SHR mesenteric arteries a larger innervation was also observed in accordance with previous studies in the same vessel [223, 255]. When comparing FPH model to essential hypertension it is visible that innervation is larger in MUN and CONTROL rats. It is important to notice that, at the age of 6 months, MUN rats did not differ in weight relative to CONTROL rats and the same was observed between SHR and WKY. However, at the same age, the SHR are smaller than MUN rats. This is likely related to the fact that they originate from different strains (WKY and Sprague Dawley, respectively) and Sprague Dawley rats are larger [256]. As so, it is expected that MUN rats presented larger vessels and, therefore, denser sympathetic innervation. This fact is also compatible with the larger adventitial layer, probably due to the trophic effects of NA.

In order to confirm that a larger innervation was associated with higher NA release, functional studies were conducted using radioactive NA. In sympathetic terminals, [^{3}H]-NA can be taken up by a specific NA transporter into vesicles [257, 258]. Therefore, total tissue tritium content (per mg of tissue) can be considered an indicator of the sympathetic innervation density [233]. The total tissue content of the mesenteric arteries of MUN was significantly higher than in CONTROL, indicating that the nervous terminals of MUN can incorporate more NA. Also, this is compatible with an increased bioavailability of NA in the synaptic cleft of MUN rats. Similar data was found in SHR as in previous studies with the mesenteric [233] and tail artery [259] and also mesenteric resistance arteries [260].

To evaluate possible differences between blood vessels, the mesenteric vein and the tail artery were also studied. These vessels have been extensively used as models for the study of neuromodulation exerted by substances in the vasculature [222, 233, 261]. This work evidence that hyperactivation of SNS is a generalized phenomenon in MUN vessels, as shown by the significant increased NA release upon electrical stimulation. It was also observed that the increase in basal and electrically induced NA release in MUN vessels was more noticeable in arteries than in veins. This data also suggests that the vascular tone would be higher in arteries from MUN than in CONTROL since NA, once released,

activates postsynaptic α_1-adrenoceptors in VSMC leading to vasoconstriction in arteries and in veins, as noticed on splanchnic circulation in animal models of hypertension [262, 263]. As so, our data is in agreement with studies demonstrating that SNS activation seem to be increased in other models of FPH [173, 178, 183, 264-266]. Indeed, there is evidence of an increased circulating levels of NA in animal models of FPH [174, 180, 267] and also in LBW humans [181]. Therefore, and in accordance with those reported data, our findings indicate that the sympathetic hyperactivity observed in MUN vascular tissues can be explained by the larger amount of sympathetic innervation, as demonstrated by TH staining. An increased sympathetic neurotransmission can contribute to increased vascular tone and to the observed remodelling, which, in turn, can be one of the mechanisms behind hypertension development in MUN.

When comparing to SHR, the increased NA release observed was analogous to MUN rats, although SHR presented a smaller basal outflow. This data is in accordance with studies previously performed in SHR, where a sympathetic hyperactivity was also described in cerebral artery [268], tail artery [26] and mesenteric bed [222].

The elevated sympathetic neurotransmission in MUN and SHR support that SNS play a role in the development of hypertension in both fetal programming and essential hypertension, supporting the neurogenic hypothesis of hypertension [71].

Fetal undernutrition is associated with a sympathetic hyperinnervation in the adventitia layer of mesenteric artery.

Fetal undernutrition is associated with a sympathetic hyperactivation and enhanced sympathetic neurotransmission in several vascular beds.

FPH and essential hypertension exhibit similar alterations in vascular sympathetic neurotransmission.

iv. RAS ALTERATIONS IN FETAL PROGRAMMING OF HYPERTENSION

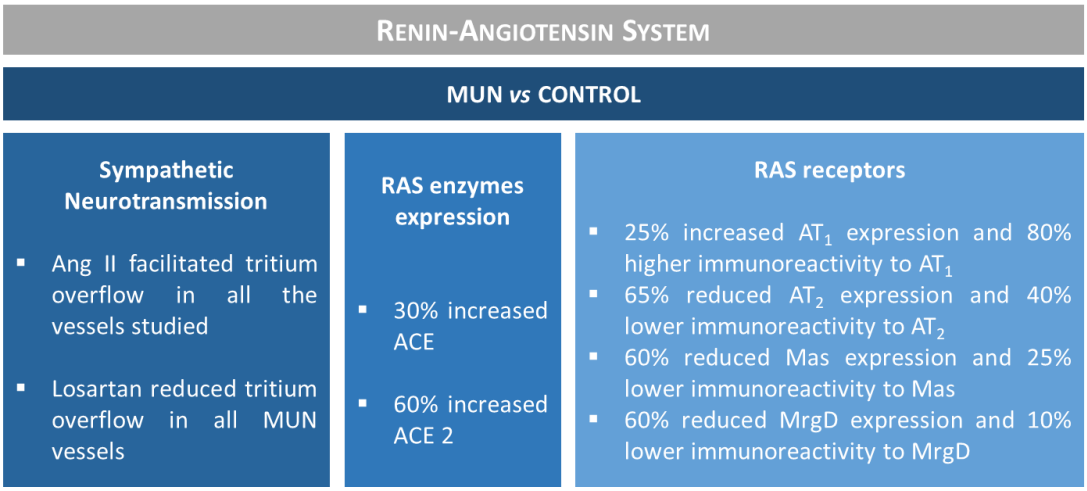

FIGURE 56| RAS alterations in MUN rats.

A presynaptic functional alteration of the perivascular sympathetic neurotransmission was found in the rat model of FPH, which could contribute to the development or maintenance of hypertension. Sympathetic activity is regulated at the presynaptic level through auto-receptors, and it is also influenced by several other factors locally released, including adenosine, prostaglandins and Ang II [269-271]. Ang II may play a key role in the observed alterations in sympathetic neurotransmission, since functional cross-talk between AT_1 receptors and other vascular signalling pathways is believed to contribute to hypertension and remodelling [47, 247].

The RAS has been found to be altered in several animal models of fetal programming. Thus, it has been shown that inappropriate activation of the RAS may occur in response to prenatal exposure to GC, which up-regulates expression of the renal RAS [272]. In addition, central expression of the AT_1 receptors is increased in offspring exposed to maternal protein restriction [190, 236] and nicotine [193]. However, the relationship between RAS and SNS has not been addressed, particularly in the adventitia. Therefore, the possible influence of RAS on sympathetic neurotransmission was evaluated. For this part of the study, only the FPH model was used.

The present work demonstrates that release of NA from sympathetic nerve terminals is under the influence of Ang II. Indeed, exogenous Ang II caused an increase in NA release

both in arteries and veins from CONTROL and MUN rats, confirming the interplay between RAS and vascular sympathetic neurotransmission, through facilitatory RAS presynaptic receptors. The facilitation of NA release induced by exogenous Ang II in CONTROL rats was more pronounced in the arterial territories than in the veins. An increased facilitatory role of Ang II on NA release has been previously demonstrated in rat models of hypertension, including in SHR [273-275].

Ang II deleterious effects on the cardiovascular system are known to be mediated through AT_1 receptors [47, 247]. Therefore, we explored the role of this type of receptor on sympathetic activity. When AT_1 receptors were blockade (in the presence of Losartan, an AT_1 receptor antagonist), NA overflow was similar to that obtained in the absence of this drug in CONTROL rats. However, in MUN rats a reduction of NA overflow was observed in the presence of Losartan, indicating a blockade of tonic facilitation. This result suggest that local Ang II is being released acting as paracrine factor on presynaptic AT_1 receptors, facilitating sympathetic neurotransmission. As so, this data suggests the occurrence of higher levels of endogenous Ang II in the sympathetic cleft of MUN rats. Curiously, the tonic effect, mediate by endogenous Ang II on presynaptic AT_1 receptors was similar in all the MUN vessels studied, discarding the possibility that this vascular sympathetic alteration occurs only locally or in specific territories, but rather indicating a widespread effect both in resistance and capacitance vessels.

The measurement of Ang II in the synaptic cleft was not possible, due to methodological limitations. Nevertheless, plasmatic Ang II was addressed and no significant difference was detect between MUN and CONTROL. Yu et al. reported reduced plasma levels of Ang II in male adult offspring of GC-treated pregnant rats [276]. However, in a maternal GC-exposed sheep model, circulating levels of Ang II in 6 months old offspring were not significantly changed [277]. As described in the literature, plasmatic Ang II levels do not always reflect local Ang II levels, which may have more relevant effects on specific organs. To assess local Ang II production, it has been proposed that local ACE gene expression or activity should be used [278, 279]. In fact, previous data provide evidence that increased vascular ACE lead to increased local Ang II formation [278, 280]. Therefore, the presence of RAS enzymes in the vascular wall was investigated in MUN rat model of FPH. Since all the vessels studied showed a similar functional profile in

vascular neurotransmission, the mesenteric artery was chosen as a representative vessel for this study.

Renin expression was not different in MUN vessels. It is known that vascular angiotensin generation depends on the uptake of circulating renin and/or its precursor prorenin [281]. This uptake into the vascular wall may be an important mechanism to enhance Ang II-dependent vascular tone or growth [280]. However, both ACE and ACE2 exhibited a higher expression in the mesenteric arteries from MUN rats. Usually, lower levels of ACE2 expression are found in VSMC and in the adventitia of large blood vessels [282]. This was also observed in mesenteric arteries from CONTROL rats. It has been proposed that the increase in tissue levels of ACE2 in pathological conditions may reflect a compensatory response, which may contribute to alter the balance of Ang II and Ang 1–7 pathways within a particular tissue or cell type [283]. This is also evidenced by the fact that the circulating levels of ACE2, which are typically low or even undetectable in physiological conditions, are also increased in experimental conditions of diabetic hypertension [284]. Therefore, the fact that both ACE and ACE2 expression are increased in MUN mesenteric arteries, suggests a potential compensatory mechanism, which would increase Ang II degradation into Ang 1-7.

Based on the above results, it seems plausible that the higher inhibition of sympathetic neurotransmission by AT_1 receptors blockage with Losartan in MUN rats is related to larger AT_1 receptor content. Therefore, their expression in the mesenteric artery and their distribution, specifically in the adventitia layer of the artery, were evaluated. In fact, a higher expression of AT_1 receptors was demonstrated in MUN mesenteric arteries when compared to CONTROL rats. This was corroborated by LSCM images which show higher immunoreactivity for AT_1 receptors and localization of these receptors within sympathetic nerves. This fact can explain, at least in part, the facilitatory tone regulating NA release from MUN mesenteric arteries stimulated with exogenous or endogenous Ang II. Together with functional data, these results reveal and support that enhanced sympathetic neurotransmission is related to alterations in AT_1 receptors in MUN rats. These results are in line with previous works that identify an upregulation of AT_1 expression in the kidneys from maternal undernutrition rat offspring [169], and in the brain of adult rats exposed to low protein diet [236] or to nicotine [193] during fetal

development. Similarly, increased pulmonary AT_1 receptor expression was found in lungs of mice exposed to hypoxia [110] and in the kidney of near-term ovine fetuses exposed to high altitude [285]. Our data add support to the implication of alterations in AT_1 receptors in FPH.

Besides, lower expression of AT_2 receptor was found in the mesenteric artery of MUN rats. Downregulation of AT_2 receptors has also been demonstrated in the kidney of offspring exposed to maternal undernutrition and low protein diet [171, 286], hypoxia [285] and GC [114].

Regarding the ACE2/Ang 1-7/Mas and MrgD axis, a lower expression and immunoreactivity for Mas and MrgD receptors were also observed in mesenteric arteries from MUN rats. Similar results are found in Mas receptor protein expression, being also significantly lower in the brain of a 6-month sheep exposed to maternal GC [287]. The same group demonstrated that the reduced expression of the Mas receptor and increased metabolism of Ang 1–7 in brain may contribute to the loss of Ang 1–7 tone, as well as the enhanced responsiveness of the Ang II-AT_1 receptor pathway in GC-dependent programming. The present data support similar findings in the vascular wall from MUN rats. The potential increase in Ang 1-7 by the enhanced ACE2 expression would be blunted by the reduction in receptor expression.

Considering the actions of Mas, MrgD and AT_2 receptors activation, favouring the vasodilation and natriuresis and the opposite effects of AT_1 receptor activation [42, 47, 51, 283], our results support that mesenteric artery from MUN rats is more prone to vasoconstriction, inflammation, and oxidative stress. Ang II has been shown to induce fibrosis and hypertrophy through AT_1 receptor activation [47, 247]. Therefore, the present data suggest that, in addition to an altered sympathetic neurotransmission, RAS alterations may also contribute to the observed remodelling in mesenteric arteries. In this study, the postsynaptic function was not assessed. However, it is likely that vasoconstriction may be also enhanced. This is also supported by the fact that we observed inward remodelling and it has been suggested that this may be induced by sustained vasoconstriction [288-290] or reduced blood flow [30, 291]. Altogether, the observed alterations in RAS and SNS can contribute to the development of hypertension in MUN rats.

The present data reveal for the first time the presence of RAS receptors not only in sympathetic nerves but also in other adventitia cells. Some of these cells seem to be Schwann cells. This possibility was confirmed by data showing that AT_1 receptors were present in GFAP positive cells, a marker for glial cells such as Schwann cells. In fact, the presence in Schwann cells of other receptors or enzymes with a role on NA neurotransmission was previously demonstrated by our group [26, 223], supporting a trophic role of these cells and of its close association with neurons. These data suggest that Ang II may be exerting trophic actions through AT_1 receptors, contributing to the hypertrophy of sympathetic innervation, observed in MUN rats. In addition to the location of these receptors on Schwann cells, confocal images also show that these receptors are present in other adventitia cells, possibly macrophages, mesenchymal cells or fibroblasts. Activation of fibroblasts may be responsible for proliferation and the increased collagen deposition in MUN mesenteric arteries. Indeed, the adventitia has been identified as an important source of ROS that may function as paracrine molecules modulating neighbouring cells and, thus, contributing to vasoconstriction and vascular remodelling [23]. Adventitial fibroblasts have been shown to produce substantial amounts of NADPH oxidase-derived ROS in response to vascular injury or vasoactive substances, and play an active role in collagen deposition and vascular remodelling [292]. Among them, Ang II is recognized as one of the key humoral factor implicated in NADPH oxidase activation in the vascular wall [90, 293, 294]. The present data also support this mechanism in MUN rat mesenteric arteries by showing a higher immunoreactivity to p22-phox, the stabilizing subunit of NADPH oxidase, which is the main enzymatic system responsible for superoxide anion in the vasculature [85, 293].

An increased level of ROS, if not counteracted by enough antioxidants, may led to oxidative damage. In fact, in the MUN model of FPH, our group has previously demonstrated that at the age of 6 months MUN rats exhibit lower levels of plasmatic antioxidants [204]. Higher expression of NADPH oxidase and Xanthine oxidase have also been reported in the heart of these animals [220]. The presence of higher NADPH oxidase in the vasculature of this model of FPH adds evidence of the importance of oxidative stress in FPH [168]. An increased ROS production in the vascular wall, can contribute to hypertension in various ways as through remodelling, as previously

described, and also by promoting endothelial dysfunction. In this regard, previous data suggested that superoxide anion release from the adventitia might reduce endothelial NO bioavailability [27, 295], which would also enhance vasoconstriction and increase resistance. Besides, excess ROS may be also implicated in the observed increase in sympathetic neurotransmission in MUN mesenteric arteries. Interestingly, it has been demonstrated that an increased ROS is a primary mechanism whereby Ang II causes an increased SNS activity, in a complex mechanism involving the contribution of the adaptive immune response [296].

Taking together, the present data indicate that increased Ang II/AT_1 receptor axis in the adventitia may be implicated in the development of vascular remodelling, fibrosis and enhanced sympathetic neurotransmission in MUN rats, being ROS possible mediators.

Ang II is a hydrophilic substance and has been mainly regarded as a humoral factor with activity on membrane receptors. However, the present work demonstrated, by confocal microscopy, the presence of nuclear location of RAS receptors. The presence of RAS receptors in cellular nucleus has been previously described in the literature. Robertson and Khairallah reported over 40 years ago the localization of Ang II binding sites on the chromatin fraction of VSMC and cardiomyocytes suggesting an intracellular site of action for Ang II [297]. Several studies subsequently identified Ang II receptors using classical receptor binding techniques on nuclei isolated. In rat models it was reported the existence of nuclear AT_1 and AT_2 receptors in the kidney, liver and brain [298]. A nuclear localization of Mas receptor was also find in VSMC from the kidneys of young adult sheep [299]. The present study, demonstrating nuclear RAS receptors clearly support an emerging view for the localization of various GPRCs to the nucleus [298, 300, 301]. Further studies are needed to clarify the impact of nuclear GPCRs in pathological conditions to evaluate their relevance as putative therapeutic targets.

Fetal undernutrition involves a tonic facilitation mediated by Ang II, trough AT_1 receptors, leading to an increased vascular sympathetic activity.

Fetal undernutrition may enhance the production of Ang II and Ang 1-7 in the mesenteric artery inducing ACE and ACE2 expression.

Fetal undernutrition also alters the expression of RAS receptors, favouring the vasoconstrictor, inflammatory, fibrotic and oxidative role of Ang II in AT_1 receptors, thus contributing to an elevation of BP.

The presence of nuclear RAS receptors clearly support an emerging view for the localization and signaling pathways of GPCRs in the nucleus.

Fetal undernutrition is associated with an oxidative damage in the adventitia of mesenteric artery possible through an increase in ROS production together with a decreased antioxidant capacity. ROS production through Ang II actions may participate in vascular hypertrophy and fibrosis observed in rats exposed to fetal undernutrition.

The present work points out the tunica adventitia has an important layer in the development of FPH. The physiologic function of the adventitia was believed to be limited to providing structural support to the blood vessel wall, transporting oxygen and nutrients to the adventitia and outer media of large vessels, and maintaining sympathetic innervation of the vessel wall. These were generally considered physiological static functions and were not believed to play a dynamic role in vascular development and vascular injury and repair [24]. However, the tunica adventitia is the most complex layer of the vessel wall and is comprised of a variety of cells including fibroblasts, immunomodulatory cells (dendritic and macrophages), progenitor cells,

vasa vasorum, endothelial cells, adrenergic nerves and perivascular adipose tissue. As a result, this layer plays important roles in the vascular development and remodelling. In response to vascular stress or injury, adventitial cells are often the first to be activated and re-programmed to then influence tone and structure of the vessel wall, to initiate and perpetuate chronic vascular inflammation, and to act to stimulate expansion of the vasa vasorum, which can act as a conduit for continued inflammatory and progenitor cell delivery to the vessel wall [23]. Under conditions of elevated BP, the adventitia becomes the predominant wall component due to the profound influence on intima and media function in disease [22, 23]. As so, our work corroborates others suggesting that the adventitia exerts a modulatory role on vascular function [27]. Thus, a comprehensive investigation of the constituents of the adventitia, both in normal physiology and in disease, is needed to understand how they interact and cross-talk to promote and inhibit vascular disease [24].

In summary, this study supports the existence of vascular remodelling in MUN mesenteric arteries that contribute to increased vascular resistance and subsequent increase in BP. Our data also supports the occurrence of a sympathetic hyperactivation, involving a tonic facilitation, by endogenous Ang II, through presynaptic AT_1 receptors. Moreover, the alterations in the expression of vascular RAS receptors and enzymes demonstrated in this work can justify, at least in part, the occurrence of hypertension in these animals. In particular, for the first time in an adult murine model, it is demonstrated that an increased vascular sympathetic activity secondary to an inappropriate RAS activation, may play an important role on hypertension development subsequent to fetal undernutrition (Figure 57). Future studies will establish the role of oxidative stress in mediating the abnormal and early interplay between RAS and SNS.

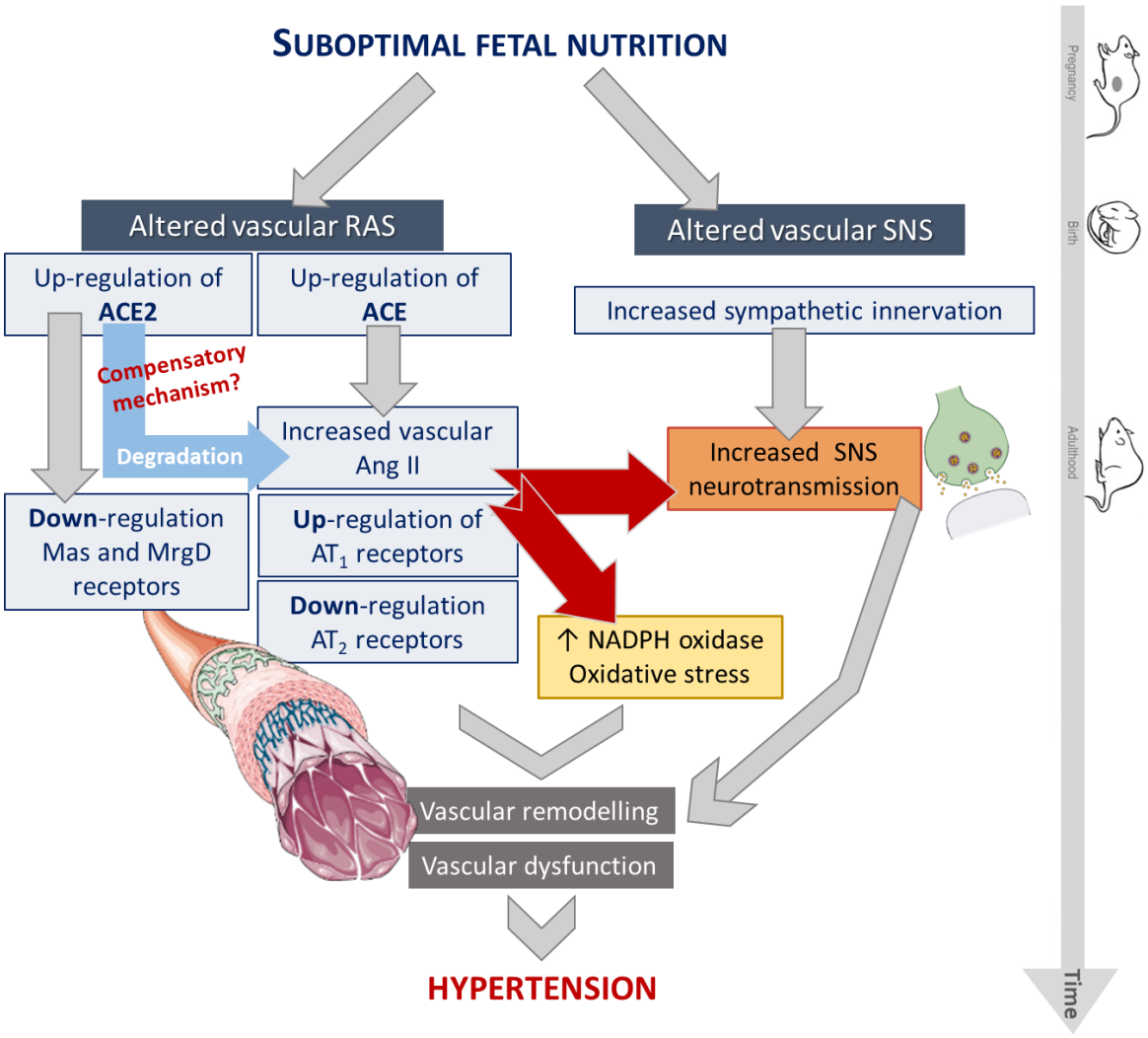

FIGURE 57| Representative scheme of the most relevant findings of this Thesis.

Conclusions

Conclusiones

CONCLUSIONS

i. Fetal undernutrition leads to high BP in adult males rats without alterations in HR.

ii. Hypertension induced by fetal undernutrition is milder compared to the essential hypertension model SHR, possibly related to the absence of tachycardia.

iii. Fetal undernutrition induces inward hypertrophic remodelling in the mesenteric artery, with the contribution of both the media and the adventitia layers, and the presence of vascular fibrosis.

iv. Hypertension programmed by fetal undernutrition and essential hypertension share similar characteristics of vascular remodelling in the mesenteric artery.

v. Fetal undernutrition is associated with hypertrophy of the sympathetic innervation in the mesenteric artery and enhanced sympathetic neurotransmission in several vascular beds.

vi. FPH and essential hypertension exhibit similar alterations in vascular sympathetic neurotransmission.

vii. Increased vascular SNA in rats exposed to fetal undernutrition may be, at least in part, mediated by tonic facilitation by Ang II, trough AT_1 receptors.

viii. Local Ang II and Ang 1-7 production in the mesenteric artery may be enhanced in rats exposed to fetal undernutrition, show by the higher ACE and ACE2 expression.

ix. Fetal undernutrition is associated with RAS receptors disbalance, with a higher AT_1 and lower AT_2, Mas and MrgD receptors, favouring the vasoconstrictor, inflammatory, fibrotic and oxidative role of Ang II.

x. FPH is associated with increased NADPH oxidase on adventitial cells, possibly related to Ang II through AT_1 receptors on these cells.

xi. Adventitial ROS production through Ang II actions may participate in vascular hypertrophy and fibrosis observed in rats exposed to fetal undernutrition.

General conclusion: In rats exposed to fetal undernutrition increased vascular sympathetic neurotransmission contributes to hypertension development, through enhanced NA spill-over and vascular remodelling, alterations in common with essential hypertension. In fetal programming, enhanced sympathetic activity is caused, at least in part, by adventitial Ang II production and imbalanced expression of RAS receptors.

CONCLUSIONES

i. La desnutrición fetal conduce a hipertensión en ratas macho adultas sin alteraciones en la frecuencia cardíaca.

ii. La hipertensión inducida por la desnutrición fetal es más leve en comparación con el modelo de hipertensión esencial, rata SHR, posiblemente relacionado con la ausencia de taquicardia en el modelo de programación fetal.

iii. La desnutrición fetal induce remodelado hipertrófico de tipo "inward" en la arteria mesentérica, con la contribución de las capas de media y adventicia, y la presencia de fibrosis vascular.

iv. La hipertensión programada por la desnutrición fetal y la hipertensión esencial comparten características similares de remodelado vascular en la arteria mesentérica.

v. La desnutrición fetal se asocia con hipertrofia de la inervación simpática en la arteria mesentérica y neurotransmisión simpática elevada en varios lechos vasculares.

vi. La hipertensión programada por la desnutrición fetal y la hipertensión esencial muestran alteraciones similares en la neurotransmisión simpática vascular.

vii. El aumento de la actividad simpática vascular en ratas expuestas a la desnutrición fetal puede estar, al menos en parte, mediado por la facilitación tónica por Ang II, a través de los receptores AT_1.

viii. En ratas expuestas a desnutrición fetal la producción local vascular de Ang II y Ang 1-7 puede estar aumentada en la arteria mesentérica, como sugiere la mayor expresión de ACE1 y ACE2.

ix. La desnutrición fetal se asocia con el desequilibrio de los receptores del SRA, con mayor expresión de AT_1 y menor de AT_2, Mas y MrgD, lo que favorece la actividad vasoconstrictora, inflamatoria, fibrótica y oxidativa de la Ang II.

x. La hipertensión programada por la desnutrición fetal está asociada con un aumento de la NADPH oxidasa en las células de la adventicia, posiblemente mediada por Ang II a través de receptores AT_1 localizados en estas células.

xi. En ratas expuestas a desnutrición fetal, el aumento en la producción de ROS en la adventicia puede participar en el desarrollo de hipertrofia y fibrosis vascular, a través de la Ang II.

Conclusión general: en las ratas expuestas a la desnutrición fetal, el aumento de la neurotransmisión simpática vascular contribuye al desarrollo de la hipertensión, a través del aumento de la liberación de noradrenalina y el remodelado vascular, alteraciones en común con las observadas en el modelo de hipertensión esencial. En la programación fetal, la actividad simpática aumentada es causada, al menos en parte, por la Ang II y el desequilibrio en l expresión de los receptores del SRA.

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Appendix

Articles published during this Thesis

Insights in sympathetic nervous system and GPCRs interplay in fetal programming of hypertension: a bridge for new pharmacological strategies

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TEASER

The interaction between sympathetic nervous system and G-protein coupled receptors is a key factor to discover new pharmacologic interventions to prevent/treat fetal programming of hypertension.

ABSTRACT

Cardiovascular diseases (CVDs) are the first cause of death owing to non-communicable diseases worldwide. In addition to the classical CVDs risk factors related to lifestyle/genetic background, exposure to adverse intrauterine environment compromises fetal development leading to low birth weight and increasing offspring susceptibility to develop CVDs later in life, particularly hypertension, a process known as fetal programming of hypertension (FPH). In FPH animal models, permanent alterations were detected in gene expression, in the structure and function of heart/blood vessels, compromising cardiovascular physiology and favoring hypertension development. This review focuses on the role of sympathetic nervous system and its interplay with G-protein coupled receptors, emphasizing strategies that envisage the prevention/treatment of FPH through interventions in early life.

Cardiovascular diseases (CVDs) are the main cause of mortality/morbidity due to non-communicable diseases worldwide, and more than 75% of deaths occur in low/middle-income countries [1]. Together with the already known CVDs risk factors related to lifestyle (sedentary, unhealthy diet, tobacco) and genetic background, it is well recognized the importance of fetal life [2-4]. The fetus can adapt to adverse intrauterine conditions, as malnutrition, oxygen deprivation, placental insufficiency, exposure to excess glucocorticoids (GC) or toxic substances, to ensure survival [5-7]. However, these conditions induce alterations in fetal development, which result, later in life, in an increased susceptibility to develop CVDs, a phenomenon named Developmental Origins of Health and Disease (DOHaD) or fetal programming.

David Barker was the first to advocate the hypothesis that adaptive responses of the fetus to maternal undernutrition (MUN) resulted in low birth weight (LBW) and, in adult life, in the development of coronary heart disease and hypertension [2, 8]. Over the past years, several epidemiological studies in populations exposed to starvation/intrauterine stress have validated Barker´s hypothesis, demonstrating an association between LBW and increased blood pressure (BP) in adult life, a process known as fetal programming of hypertension (FPH) [6, 9-11].

MUN seems to be the major cause of LBW in underdeveloped or developing countries, while in high-income societies LBW is mostly related with prematurity or obstetric complications associated with delayed pregnancy. For example, placental insufficiency, causing placental hypoxia and fetal oxygen delivery privation [12, 13], and pre-eclampsia, are conditions related with the increased maternal age [14, 15], associate with offspring hypertension in adult life [15]. Excess fetal exposure to GC may also contribute to LBW [16], which is clinically relevant, since women with risk of preterm delivery are treated with CG to improve fetal pulmonary function and reduce mortality. Besides, maternal psychological stress may abnormally elevate cortisol levels [17]. Cortisol access to the fetus is limited to the placental barrier owing its conversion by 11-β hydroxysteroid dehydrogenase 2 into cortisone. However, this enzyme can be reduced in adverse fetal conditions [18], favoring cortisol access to the fetus. Other stress factors, such as exposure to toxic substances (alcohol, tobacco) or environmental pollutants, can also affect fetal development, contributing to FPH [19-21].

Despite the efforts carried out so far, the mechanisms underlying the predisposition to FPH are not completely understood. Nevertheless, increasing evidence has shown the implication of oxidative stress [22, 23], renin-angiotensin system (RAS) [24-26] and sympathetic nervous system (SNS) [27-29], as well as alterations in the GC axis [6, 30-

32]. Some of these alterations seem to be mediated by epigenetic modulation of genes implicated in cardiovascular control [33-36]. Altoghether, these aspects may contribute to the FPH development (Figure 1).

Experimental data gathered in animal studies have been very valuable to understand the relationship between adverse intrauterine environment and increased susceptibility to hypertension. Among others, the most common animal models mimicking the different fetal stress factors are maternal nutritional restriction during pregnancy or lactation (protein, global nutrients, vitamins, etc), exposure to hypoxia, placental insufficiency or treatment with GC [37, 38].

In these animal studies, high BP is associated with changes in blood vessels (remodeling, stiffening and endothelial dysfunction), heart (cardiomyocyte hypertrophy or intramyocardial fibrosis), and kidneys (low nephron number, alterations in sodium handling, etc.) [6, 39-42]. These studies have also evidenced the important influence of sex in FPH. As so, male offspring from all the above-mentioned experimental models develop hypertension by adult age, while females remain normotensive or develop milder forms of hypertension [43]. This sexual dimorphism has been proposed to be related to the protective effects of estrogens on the cardiovascular system [44, 45]. In addition, the concept of sex of the placenta has also been put forward, and it has been suggested that the placenta nourishing female fetus could adapt better to adverse intrauterine environments, thus, contributing to the lower impact of FPH in females [46, 47]. Despite the fact that in animal models of FPH the role of sex has been well documented, in humans, this is not so evident. This discrepancy is likely related to differences in the age of the populations under study, and the confounding factor of menopause [44, 48].

Sympathetic Nervous System and Fetal Programming of Hypertension

SNS has an integral role in the regulation of heart rate and contractility, vascular tone and fluid volume. Excessive sympathetic nerve activity (SNA) has deleterious effects namely increased cardiac output, peripheral resistance, retention of salt and water, thus, contributing to the development and/or maintenance of hypertension [49-53]. In vessels, increased SNA is responsible for vascular remodeling related to smooth-muscle hypertrophy and fibrosis [54].

Data from humans and experimental animal models of hypertension support the neurogenic hypothesis of hypertension, which considers SNA as the primary factor underlying the elevation of BP [55-58]. Although the main causes of increased SNA

remain undefined, alterations of reflexes and/or metabolic factors have been proposed as candidates [58].

In several animal models of FPH, altered SNS control is evident. In offspring of maternal low-protein-diet (MLPD) rats, hypertension was associated with increased cardiovascular sympathetic tone [59], while no baroreflex dysfunction was found [60]. Offspring from rats exposed to intermittent hypoxia at the end of pregnancy exhibit increased BP related to an altered sympatho-vagal balance [61]. Also, in MLPD rat offspring, increased SNA was reported in response to physical stress induced by activation of exercise pressor reflex [62], most probably due to an exaggerated increase in renal SNA [63]. Other FPH rat model (induced by hypoxia *in utero*), showed an increased muscle SNA and an hyper-sympathetic innervation in skeletal muscle arteries [28].

Another alteration corroborating an abnormal SNS control in FPH rats is the elevation of catecholamines in the cardiovascular system, due to increased release or biosynthesis. In FPH rats induced by placental insufficiency or MLPD, circulating catecholamines were raised both in the fetus and in adult offspring, possibly because of increased SNA [29, 64, 65]. In this sense, an increased electrically-evoked noradrenaline output has been reported in mesenteric arteries from MUN rat offspring [66]. Besides, there is evidence of elevated catecholamine biosynthesis due to increased expression of adrenal phenylethanolamine N-methyltransferase (PNMT), in dexamethasone exposure during gestation rat offspring [67]. Elevated PNMT mRNA expression was also detected in spontaneously hypertensive rat (SHR) brain [68].

In addition to alterations at the peripheral level, data also indicates changes in central SNA. An elevated basal and stimulated dopamine output has been described in rat offspring exposed to prenatal stress [69]. Significant SNA alterations were also found in *nucleus tractus solitarius* [62], hippocampus and hypothalamic paraventricular nucleus from MUN rat offspring, with a marked activation of hypothalamo-pituitary adrenal axis [30]. Changes in central SNA could be related to alterations in redox balance. Oxidative stress is known to contribute to FPH, though many mechanisms (for details, see review [23]), but the relevance of SNA in redox status and *vice versa* has been recently highlighted. For example, in SHR, oxidative stress contribute to an increased SNA [70], and MUN rat offspring exhibit changes in brainstem redox status accompanied by an increase in SNA [71].

Excess cortisol levels may also contribute to FPH through the SNS. For example, in sheep fetuses, it has been shown that adrenal GC contribute to cardiovascular regulation by modulation of SNA and arterial baroreflex [72].

In LBW humans, there is also evidence of altered SNA [73, 74]. Children with LBW, due to prematurity or small for gestational age, excrete high catecholamines levels [75], which has been associated with longer periods of tachycardia during stress exposure [76]. LBW is also associated with exaggerated BP responses to psychological stressors, related to alterations in baroreflex control [77], and greater SNA response [76]. Young men and women born with LBW also exhibit raised muscle SNA [73] and exaggerated sympathetic discharge in response to muscle metaboreflex [78]. Abnormalities in heart rate and baroreflexes are also observed in premature infants, related to the immaturity of their autonomic nervous system [79], which may contribute to increased cardiovascular risk in this population [80].

Taken together, data are consistent with a pathophysiological role of the SNS in FPH, with evidence indicating the occurrence of an increased SNA.

G-Protein-Coupled Receptors and FPH

G Protein-Coupled Receptors (GPCRs) are a large family of seven-transmembrane domain proteins activated by several peptides or proteins, such as neurotransmitters and hormones [81]. GPCRs trigger intracellular signal transduction pathways and influence several physiological functions, including cardiovascular control [82]. Indeed, some GPCRs are involved in the modulation and regulation of vasodilator and vasoconstrictor responses, thus, influencing vascular responsiveness and BP control.

Dysregulation of GPCRs-mediated mechanisms during fetal life may be implicated in FPH. Among possible mechanisms, we can include changes in the receptor itself, or in the intracellular mechanisms or even in the concentration of receptor ligands. All these changes can lead to an up or down regulation of GPCRs, which may modify endocrine and nervous responses involved in BP control.

Below we discuss evidence of alterations of key GPCRs, namely adrenoceptors, angiotensin, adenosine and endothelin receptors in FPH (Figure 2).

Adrenoceptors

Noradrenaline released from sympathetic nerves can activate α– or β–adrenoceptors modulating postsynaptic nerve or vascular responses with both types described as being involved in FPH. Indeed, studies in rats provide evidence of the implication of α–adrenoceptors localized in central SNS in FPH: the natriuretic effect induced by central noradrenaline injection was abolished by α_1-adrenoceptor antagonist (prazosin) in MLPD rat offspring [83]; administration of α_2-adrenoceptor antagonist (yohimbine) normalized urinary sodium excretion and reduced BP in FPH offspring [83]. Conversely, β-adrenoceptors may also contribute to FPH: in 2-week-old MUN rat offspring, the myocardial mRNA expression of β_2-adrenoceptors was increased, which might explain the increased susceptibility to ischemia-reperfusion injury [84].

In humans, recently, the involvement of adrenoceptors in FPH has been suggested since α-adrenoceptors blockade attenuated the development of hypertension in preeclampsia [85].

Taken together, information is in agreement with a relevant role of adrenoceptors in FPH.

Angiotensin Receptors

Contribution of angiotensin receptors (ATR) to FPH has been demonstrated in animals exposed to nutritional insults such as low-protein, high-fat or sugar diets [86-88], toxics, such as nicotine [89, 90], or to CG [91].

Accumulating evidence in rats indicates that AT1R and AT2R expression is altered in response to several fetal stressors. An upregulation of AT1R expression was found in the kidneys [24] and mesenteric vasculature [66] from MUN rat offspring and in the brain of adult rats exposed to MLPD [92] or to nicotine [90] during fetal development. Similarly, increased pulmonary AT1R expression was found in lungs of mice exposed to hypoxia [93] and in the kidney of near-term ovine fetuses exposed to high altitude [94]. Regarding AT2R expression, a downregulation was shown in the kidney of offspring of MUN, MLPD [26, 95], hypoxia [94] and fetal exposure to GC [96].

Epigenetic and gene expression changes are responsible for many alterations in FPH [36], some of them associated with RAS. MLPD led to an increased AT1R gene expression and subsequent upregulation of AT1R in adrenal glands from adult rat offspring, ultimately contributing to the development of hypertension [35]. Maternal GC in early pregnancy may induce changes in methylation and expression of the AT1R gene. In addition, in prenatal high sucrose exposure aged offspring, mRNA and protein expressions of AT1R gene were significantly increased in large/small blood vessels,

which should be closely associated with the changes of epigenetic mechanisms such as histone modifications [97]. In brain MLPD mice offspring, an upregulation of mRNA expression of angiotensinogen and angiotensin-converting enzyme (ACE) and a downregulation of mRNA levels of AT2R were reported [98].

ACE is other RAS player modified in FPH. Both pulmonary and plasma ACE activities seem to be increased in MUN adult rat offspring [99].

In experimental FPH, hypertension was controlled after treatment with ACE inhibitors or ATR antagonists [25, 99, 101] similarly to what occur in essential hypertension.

The above studies evidence the role of the classical ACE-Angiotensin II (Ang II)-AT1R/AT2R axis in FPH. Recently, the non-classical ACE2-Ang-(1–7)-Mas receptor axis was also suggested to be implicated [102], as shown by the altered Ang-(1–7) system within the brain and in the kidney in rats exposed GC in fetal life, proving their contribution to cardiovascular dysfunction.

In humans exposed to adverse intrauterine conditions, information is still scarce regarding ATRs or other RAS players. Nevertheless, children born from preeclamptic pregnancies have increased circulating levels of aldosterone [103]. Also, boys with LBW exhibited a significant increase in ACE activity and circulating Ang II, which were associated with increased systolic BP [104]. However, this association was not found in girls, supporting the existence of sex differences in FPH also in humans. Indeed, RAS alterations associated with FPH seem to be age and sex-specific [37, 91].

Overall, experimental data are consistent and support the idea that RAS might be a common mechanism that underlies hypertension of developmental origins.

Adenosine Receptors

Considerable evidence supports the involvement of adenosine and its receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) in hypertension [105, 106]. Adenosine is a key signalling molecule that coordinate a rapid multi-cellular physiological response to hypoxia or tissue damage. Indeed, chronic systemic hypoxia in utero affects cardiovascular responses in adult rat offspring through an A_1R-mediated mechanism [107]. These rats have functional A_1R in the heart and vasculature but show attenuated adenosine release and endothelium-dependent vasodilation [107].

In humans, adenosine is determinant in pregnancy diseases associated with altered umbilical blood flow, namely preeclampsia [108, 109]. Alterations in enzymes involved in

adenosine bioavailability, such as increased placental 5'-nucleotidase and reduced placental adenosine deaminase, favour high placental adenosine levels, which coupled with an enhanced $A_{2B}R$ receptor signalling, may contribute to preeclampsia pathogenesis [110]. Furthermore, in women with preeclampsia, it has been proposed that reduced angiogenesis mediated by adenosine might be associated with the development of hypertension in the offspring [111].

Altogether, these information points for a relevant role of the adenosinergic system in FPH.

Endothelin Receptors

Endothelin 1 (ET-1), mainly released from endothelium, ~~is a peptide that~~ can activate endothelin receptors type A (ET_AR) and type B (ET_BR). These receptors exert opposite cardiac and vascular effects: ET_AR mediates vasoconstriction while ET_BR mediates vasodilation [112]. In the context of FPH, few works establish a role for ET-1 and/or its receptors.

Hypoxia [114] and MUN [39] rat offspring present an enhanced vasoconstriction to ET-1. In rat offspring exposed to fetal hypoxia, treatment with tezosentan, an $ET_{A/B}R$ antagonist, caused a significant reduction in BP [114]. The selective ET_AR antagonist ABT-627 reduced BP in FPH rats, accompanied by a disruption in ET-1 expression [115]. These studies encourage further clarification regarding the usefulness of endothelin receptors as targets to treat/prevent FPH. Despite of this, recent efforts on endothelin receptor antagonists envisage their putative use for the treatment of resistant hypertension [116].

Nevertheless, more studies are required to completely understand the implication of GPCRs in the development and maintenance of hypertension of fetal origins.

Interplay between the Sympathetic Nervous System and GPCRS in FPH

SNA can be influenced by different GPCRs, since these receptors can modify noradrenaline bioavailability in the synapsis and/or interact with signaling cascades. Furthermore, altered SNA can also influence GPCRs activation by modifying their expression or conditioning its own signaling events. In FPH, increased SNA has been associated with hyper-sympathetic innervation and/or with alterations in specific GPCRs expression or function. These changes condition vascular responsiveness or renal

function and may contribute to increase BP (Figure 3). The association between FPH animal models and the experimental results related to SNS and GPCRs are depicted in Table 1.

Interventions to counteract FPH

Understanding the causative mechanisms of FPH opens new opportunities and challenges for prevention through early life interventions. Firstly, pharmacological treatments or lifestyle changes during pregnancy may help to reduce programming. Secondly, early interventions in the offspring during key developmental windows, such as lactation, may also help to reduce the impact of CVDs in the future generations.

Pharmacological or nutritional interventions with antioxidants are a plausible strategy since oxidative stress is one of the underlying mechanisms in FPH, common to different fetal stressors, namely malnutrition [117], exposure to toxic substance [118, 119] or GC [120]. Studies in animal models have provided proof of the effectiveness of antioxidant treatments, in pregnancy, against FPH. In hypoxic pregnancies, treatment with vitamin C improved transplacental oxygenation [124]. In addition, melatonin and N-acetylcisteine administration during the entire pregnancy and lactation have demonstrated effectiveness against the development of hypertension in FPH rat model induced by NG-nitro-L-arginine-methyl ester during gestation [125]. Resveratrol has also promising effects for the treatment of feto-placental flow alterations in preeclampsia, due to its anti-inflammatory/antioxidant properties in vitro [126]. In MLPD rat offspring, resveratrol administration during pregnancy partially prevents oxidative stress and metabolic dysfunction [127]. In the same animal model, prenatal treatment with lazaroid, a lipid peroxidation inhibitor, abolished the increased Ang II-mediated contractile response and prevented the increase in BP [128]. Also, tempol avoid the BP increase in adult mice of FPH induced by GC exposure [129].

In humans, pregnancy alterations such as preeclampsia, which program the offspring, are also related to oxidative unbalance. In low and middle income-countries, micronutrient dietary deficiencies can contribute to fetal programming through oxidative stress [14]. In these settings, nutritional interventions with vitamins or minerals during pregnancy have been demonstrated to be an effective approach to reduce the risk of prematurity and LBW [121] and to improve offspring cardiometabolic health [122]. In high-income societies, the main problem are the obstetric complications that alter placental function leading to LBW and premature births (and subsequent need of GC treatment). We have previous evidenced the association between a low antioxidant

status in early pregnancy and the later development of obstetric complications [14]. Therefore, antioxidant treatment can be a useful strategy to reduce preeclampsia and other placental disorders once antioxidant deficiency in pregnancy increase oxidative damage and compromise feto-placental flow through NO inactivation by reactive oxygen species. In fact, several antioxidants, namely N-acetylcisteine, Vitamin C, or melatonin, as well as NO donors, like L-Arginine, have been used in women with pregnancy complications with variable results, but most of them have shown benefits and safety [123].

The above mentioned data suggest that antioxidant therapy, either through nutritional or pharmacological interventions, may be a strategy to counteract or reduce FPH.

A second possible developmental window to counteract the effects of FPH is the lactation period. In this regard, drugs used to treat hypertension have been studied in experimental animal FPH offspring concerning their putative usefulness to modulate the development of hypertension postnatally. Administration of nifedipine (calcium channel blocker) in FPH offspring with 4 to 12 weeks did not modify BP [101], discarding the use of this pharmacological group for this purpose. However, in MLPD rat offspring and in rats exposed to placenta insufficiency, with 2-4 weeks old, captopril and enalapril (ACE inhibitors) treatment (initiated after weaning), prevented the development of hypertension, with animals remaining normotensive at least for 12 weeks after treatment suppression [25, 99, 101, 130]. This is corroborated also in MUN rat offspring, since treatment with aliskiren, or losartan, an AT1R antagonist, administered between 2-4 weeks of postnatal life, prevented the development of hypertension until 12-weeks old [131]. Treatment with AT1R antagonists such as losartan [101] or candesartan [132], were also effective in preventing the development of hypertension in MUN adult rat offspring. Candesartan not only lowered BP, but also protected against cardiac enlargement and coronary perivascular fibrosis [132]. However, this therapeutic strategy could only be implemented after birth, owing to the teratogenic effects ascribed to this group of drugs during pregnancy and their impact avoiding adequate nephrogenesis.

After birth, vitamin C and E treatments reverse the increased vasoconstrictor responses in FPH rats [133], and allopurinol, a xanthine oxidase inhibitor, had analogous effects [134]. Coenzyme Q decreased oxidative stress and DNA damage in aorta from FPH offspring [135] and resveratrol improved insulin sensitivity, vascular function and prevented cardiac dysfunction and hypertrophy [136] [137]. Therefore, antioxidant therapy should be considered, per se, or in association with other drugs in FPH.

In humans, breast milk is an important source of antioxidants and other bioactive compounds, thus, breast-feeding may help to counteract the negative effects of oxidative stress in newborns, particularly in preterm infants, which exhibit deficiency of antioxidant systems, and are at higher risk of FPH [138].

Regarding pharmacological intervention, research is needed to analyze the reverting effect of treatments on FPH with drugs binding to GPCRs. Currently, several drugs are in the forefront to be used in FPH but no ongoing clinical trials are being performed, so far, with this particular purpose.

Concluding remarks

Data collected so far suggest that FPH is related with the exposure to stress factors during pregnancy and subsequent LBW, and will likely continue to contribute to the burden of CVDs in the next decades. In this context, determining the main mechanisms implicated is imperative, namely those related with sex differences. Thus, therapeutic approaches targetted properly to these sex differences may offer the best opportunity to mitigate the progression of cardiovascular disease in both women and men. In addition, increasing evidence supports the implication of increased SNA eventually linked to some GPCRs, which modify the bioavailability of noradrenaline in the synapsis and/or interact with signaling cascades, ultimately, conditioning renal, cardiac and vascular responses.

This knowledge will open the door to risk stratification for the development and definition of new preventive/therapeutic strategies.

Conflicts of interest

The authors report no conflicts of interest.

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Figure 1: The developmental programming of cardiovascular diseases hypothesis. (Figure made with graphic components obtained from the website https://smart.servier.com)

Figure 2: Schematic representation of the involvement of adrenoceptors, angiotensin II, adenosine and endothelin receptors in FPH. (Figure made with graphic components obtained from the website https://smart.servier.com)

Figure 3: Role of SNS and its interplay with GPCRs in FPH. (Figure made with graphic components obtained from the website https://smart.servier.com)

Table 1: FPH-mediated mechanisms associated with respective GPCR and mediator levels dysregulation in experimental FPH models.

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Vascular angiotensin AT1 receptor neuromodulation in fetal programming of hypertension

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ABSTRACT

Fetal stress increases the susceptibility to cardiovascular diseases in adult age, including hypertension, a process known as fetal programming of hypertension (FPH). This study intends to investigate the interplay between vascular sympathetic nervous system (SNS) and RAS, namely the neuromodulatory role exerted by Angiotensin II (Ang II) receptor-1 (AT1) in FPH, and respective contribution for hypertension.

Methods: 6-month old Sprague-Dawley offspring from mothers fed *ad-libitum* (CONTROL) or with 50% intake during the second half of gestation (maternal undernutrition, MUN) were used. Sympathetic neurotransmission was studied in mesenteric/tail arteries and mesenteric veins by electrically-evoked [^{3}H]-noradrenaline release experiments using RAS drugs. AT1 receptors in sympathetic nerves of mesenteric arteries were investigated by immunohistochemistry and Laser Scanning Confocal Microscopy.

Results: Ang II facilitated noradrenaline release in the vessels studied from MUN and CONTROL rats. Losartan induced a tonic facilitation only in MUN vessels. Sympathetic innervation was larger in MUN versus CONTROL vessels. AT1 receptors on sympathetic nerves were present in higher amounts in MUN versus CONTROL vessels.

Conclusions: Findings support that FPH is associated with a vascular hyper-sympathetic activation, involving a tonic facilitation of prejunctional AT1 receptors by endogenous Ang II, which can justify, at least in part, the development of hypertension.

1. Introduction

Along with genetic and lifestyle factors it is now established that some stress factors during fetal life, particularly undernutrition, contribute to the development, in adult age, of cardiovascular diseases, particularly hypertension [1–8]. This is known as Fetal Programming of Hypertension (FPH). In western countries, although the probability of maternal malnutrition is low, fetal undernutrition may result from placental insufficiency and intrauterine growth retardation, which are increasing due to pregnancy delay.

Several mechanisms have been proposed to explain the relationship between poor fetal growth and later hypertension development. One of the proposed mechanisms is an alteration of the renin angiotensin system (RAS). Thus, in infants with intrauterine growth retardation, renin activity is elevated in umbilical cord and, in animal models of FPH, the pressure response to angiotensin II (Ang II) is elevated [6,9–13]. Indeed, Ang II play a crucial role in blood pressure regulation through pleiotropic actions such as vasoconstriction and vascular/cardiac hypertrophy [14]. These actions of Ang II are mediated via interaction with metabotropic cell membrane bound G-protein coupled receptors. Four types of RAS receptors are known (AT1, AT2, Mas and MrgD), of which the AT1 and AT2 receptors are the most studied. These two receptors share a similar high affinity to Ang II but the consequences of their binding are opposite. AT1 receptor is highly expressed in adults and mediates Ang II-induced vasoconstriction [15], proliferation [16], inflammation [17], and extracellular matrix remodeling [18]. On the other hand, AT2 receptor activation produces opposite effects, providing a cardiovascular protective action [19].

Ang II-induced hypertension is associated with a decrease in cardiac output and a marked increase in total peripheral resistance. Moreover, Ang II has been described to cause increased sympathetic nervous activity in rats consuming a high-salt diet [20]. However, the association between Ang II and sympathetic dysfunction in FPH based on maternal undernutrition is still to be addressed. We postulate that the effects

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mediated by Ang II are altered in FPH individuals leading to an increased vascular sympathetic activity, and consequently, to an increase in vascular responsiveness and hypertension. To check on this possibility, the interplay between SNS and RAS was studied, namely the vascular neuromodulatory role of AT1 receptor in FPH, using an animal model of FPH. We also evaluated the regional distribution/localization and relative amount of AT1 receptors and sympathetic nerves in FPH mesenteric arteries.

2. Materials and methods

2.1. Animals

2.1.1. Animal ethical considerations

Sprague–Dawley (SD) rats from the colony maintained at the animal house facility of the Universidad Autonoma de Madrid were used. All experimental procedures were approved by the Ethics Review Board of Universidad Autonoma de Madrid (CEI63–1112-A097) and conformed to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85–23, revised in 1996), the Spanish legislation (RD 1201/2005) and the Directive 2010/63/EU on the protection of animals used for scientific purposes. The rats were housed in buckets 36.5/21.5/18.5 cm (length/width/height) on aspen wood bedding, under controlled conditions of 22 °C, 40% relative humidity and 12/12 light/dark photoperiod. The animal health monitoring indicated that they were free from pathogens that may interact with any of the parameters studied. The rats were fed with breeding diet (SAFE A03) containing 51.7% carbohydrates, 21.4% protein, 5.1% lipids, 3.9% fiber, 5.7% minerals and 12.2% humidity (Safe Augy, France). Drinking water was provided *ad libitum* in all cases.

2.1.2. Experimental animal model of FPH

FPH model based on global maternal nutrient restriction was induced as previously described [21]. The litters were divided in two groups, one from mothers receiving *ad libitum* diet (CONTROL) throughout pregnancy and the other group from mothers with *ad libitum* diet during the first 10 days of gestation and 50% of the daily intake from day 11 to the end of gestation (Maternal Undernutrition, MUN). The maximum daily intake of rat chow was previously determined in a group of pregnant rats as 24 g/day. Therefore, during the second half of gestation, the second group received 12 g of rat chow per day. After delivery the litters were culled to 12 rats and the respective mothers received food *ad libitum* during the suckling period. 6 month old male offspring from MUN (n = 6) and CONTROL (n = 6) were used. The animals were euthanized using a guillotine, the method reported as the sacrificial advisable in studies involving the nervous system. The mesenteric and tail arteries and the mesenteric vein were collected and immediately used. Four segments were obtained from each vessel for different experimental procedures.

2.2. Chemicals

The following drugs were used: levo-[ring-2,5,6-3H]-noradrenaline, specific activity 44.8 Ci/mmol (DuPont NEN, I.L.C., Lisboa, Portugal), losartan (Merck Portuguesa, Lisbon, Portugal), angiotensin II (Sigma-Aldrich, St. Louis, USA) and desipramine hydrochloride purchased from Sigma-Aldrich (Sintra, Portugal). The following antibodies were used: rabbit polyclonal anti-AT1 were purchased from Santa Cruz Biotechnology, Inc., CA, USA and mouse monoclonal anti-tyrosine hydroxilase antibody (TH, ab129991,Abcam, UK) and mouse monoclonal anti-glial fribrilary acidic protein (GFAP, G6171, Sigma-Aldrich, Inc., USA). The following fluorescent probes were used: Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody, highly cross-adsorbed and Alexa Fluor 647 goat anti-rabbit IgG (H + L) antibody, highly cross-adsorbed (Molecular Probes) secondary fluorescent antibodies (Invitrogen, Life Technologies, SA, Madrid, Spain); vectashield mounting medium with DAPI (Vector Laboratories, UK). Stock solutions were made up in ultrapure water and diluted in superfusion medium immediately before use.

2.3. Hemodynamic parameters measurement

Systolic blood pressure (SBP) was measured using the tail-cuff method with a NIPREM 645 noninvasive blood pressure acquisition system for rats (CIBERTEC, Madrid, Spain). The experiments were conducted in a quiet area at 22 ± 2 °C. Firstly, the rats were placed in a chamber at 37 °C for 10–15 min to induce vasodilatation. Thereafter, they were placed inside a soft support in the darkness to prevent excessive movement. A pulse sensor and a pressure cuff were place around the tail for SBP recordings. Sessions of recorded measurements were all made by a single investigator. The cuff was inflated to 150 mmHg and 15 to 25 pressure inflate-deflate cycles were performed and data were automatically registered. The first 5 cycles were considered “acclimatization” cycles and were not used in the analysis. This procedure was obtained during 3 consecutive days for the rats to become accustomed. The measurements recorded on days 2 and 3 were similar and averaged for statistical analysis.

2.4. [^{3}H]-Noradrenaline release experiments

[^{3}H]-noradrenaline release experiments were carried out as previously described [27–29]. Vessels were preincubated in 2 mL Krebs-Henseleit solution (KHS) containing 0.1 μmol/L [^{3}H]-noradrenaline (for 60 min at 37 °C) and transferred into superfusion chambers, superfused with [^{3}H]-noradrenaline-free KHS (1 mL/min; constant rate) with desipramine 400 nmol/L to inhibit noradrenaline's neuronal uptake. After 30 min of stabilization, two periods of electrical stimulation (5 Hz, 100 pulses, 1 ms, 50 mA; Hugo Sachs Elektronik, March-Hugstetten, Germany) were applied, S_1 and S_2, with 30 min intervals (t = 90 min and t = 120 min, respectively). The superfusate was collected each 5 min period from 85 min of superfusion onwards.

To address the implication of presynaptic AT1 receptors, the agonist for AT1 receptors, Ang II (30 nmol/L), and the antagonist, losartan (100 nmol/L), were used: Ang II was added 5 min before S_2 and was present until the end of the stimulation period; losartan was added immediately after S_1 and kept until the end of the experiment.

At the end of the experiments (t = 130 min), the tritium was measured in the collected superfusate samples and in the vessels (previously solubilized: sonicated 1 h with 2.5 mL, 0.2 mol/L perchloric acid). For this, 6 mL of a scintillation mixture (OptiPhase ‘Hisafe’ 3, PerkinElmer, I.L.C., Lisboa, Portugal) was added to each sample and analyzed by liquid scintillation spectrometry (LS 6500, Beckman Instruments, Fullerton, USA).

2.4.1. Measurement of drug effects on electrically-evoked tritium overflow

Tissue labelling with [^{3}H]-noradrenaline and evaluation of electrically-evoked tritium overflow changes was performed as previously described [22,23]. Effects of drugs added after S_1 on electrically-evoked tritium overflow were evaluated as ratios of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments in which a drug was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of the respective drug).

2.5. Laser scanning confocal microscopy (LSCM) experiments

Immunohistochemistry procedures were previously described [26]. Briefly, the artery was immediately placed in cold phosphate buffer solution (PBS; in g/L): NaCl 8.0, $Na_2HPO_4.2H_2O$ 0.77, KCl 0.20, KH_2PO_4 0.19 (pH 7.2), and was cut in 4 segments. Each artery segment was longitudinally opened and fixed (paraformaldehyde 4% PBS; 50 min; room temperature). After two 15 min PBS washing cycles,

artery segments were incubated with primary antibodies raised against rabbit polyclonal angiotensin receptor subtype anti-AT1 (AT1, 1:100 dilution, overnight, 4 °C) and mouse monoclonal anti-tyrosine hydroxylase (TH, 1:100 dilution, overnight, 4 °C) to stain noradrenergic nerve terminals or mouse monoclonal anti-glial fibrillary acidic protein (GFAP, 1:200 dilution, overnight, 4 °C) to stain glial cells such as Schwann cells. Thereafter, tissues were incubated with Alexa 647 anti-rabbit and Alexa 488 anti-mouse fluorescent secondary antibodies (1:1000 dilution, 1 h, room temperature). Negative controls were incubated on adjacent sections using 10% normal horse serum or blocking solution instead of the primary antibody. After two PBS washing cycles, tissue preparations were mounted with antifading agent (vectashield mounting medium with DAPI, Vector Laboratories, UK), with the adventitial side facing up. Preparations were visualized with a Leica SP5 LSCM system (Leica Microsystems, Wetzlar, Germany) fitted with an inverted microscope (× 63 oil immersion lens). Stacks of 1-μm-thick serial optical images were captured from the entire adventitial layer, which was identified by the shape and orientation of the nuclei stained with DAPI [30].

From each preparation, three stacks of images were sequentially obtained from the same region, the first with the Ex 405 nm and Em 412–470 nm wavelength to visualize cell nuclei. The second was taken with the Ex 488 nm and Em 490–570 nm wavelength to visualize the TH or GFAP staining (location of noradrenergic terminals or glial cells) and the third with the Ex 633 nm and Em 640–720 nm wavelength, to detect the AT1 receptor distribution stained with the secondary antibody Alexa Fluor 647 (different subtypes depending on primary antibody). Image acquisition was performed always under the same laser power, brightness, and contrast conditions. Adventitia was scanned along each mesenteric artery segment and the resulting images were reconstructed separately for each wavelength.

2.5.1. Specificity of primary antibodies

The specificity of the primary AT1 antibody used has been established in previous studies: AT1 sc-31,181 [24,25]. In addition, pre-adsorption with the corresponding synthetic peptide antigen was carried out in our experimental conditions for all the primary antibodies used. Western-blot was also performed using arteries extracts as protein source to immunoblot with antibodies and/or with peptides.

2.5.2. Laser scanning confocal microscopy images quantification

Quantitative analysis of confocal z-stacks images was performed using image analysis software (PAQI, CEMUP, Porto, Portugal), as previously described [22,26]. Briefly, a sequential routine was designed and developed to analyze each fluorescent signal used. PAQI software measured the surface area and strength of the fluorescence signal marking the postganglionic nerves, the surface area and strength of the fluorescence signal marking the receptors and determined the surface area of attachment of the receptors on the nerves as well as the intensity of fluorescence of the receptors on nerves (corrected for background).

2.6. Data analysis

Results are expressed as mean ± s.e.m. and n denotes the number of animals used. Quantitative analysis of confocal images is presented as staining fractional area, expressed as a percentage of the tissue total area. Differences of means were compared for significance using one- or two-way ANOVA, followed by post-hoc Holm-Sidak's multicomparison *t*-test or Student's *t*-test. A P value lower than 0.05 was considered to denote statistically significant differences.

3. Results

3.1. Blood pressure measurements

Blood pressure was evaluated in MUN and CONTROL animals: a significantly larger SBP values were observed in MUN (164.40 ± 3.61 mmHg; n = 6) comparatively to values from CONTROL animals (134.13 ± 3.35 mmHg, respectively; n = 6; p = .003).

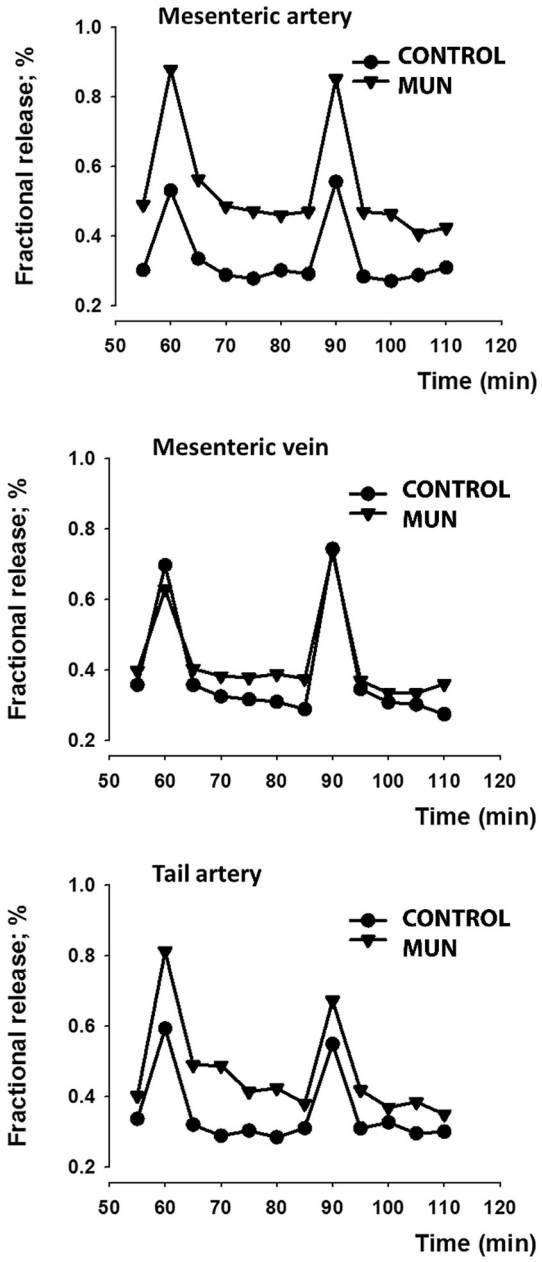

Fig. 1. Representative examples of time course tritium outflow from: mesenteric artery, mesenteric vein and tail artery from CONTROL (circles) and MUN (triangles) from typical experiments. After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period and was measured in samples collected every 5 min. Artery segments were stimulated twice by 100 pulses/5 Hz, (S_1, S_2). Each line represents the outflow of tritium from a single superfusion chamber.

3.2. Noradrenaline release from sympathetic nerve terminals

Electrically-evoked tritium overflow from tissue preparations incubated with [^{3}H]-noradrenaline has been shown to reflect action potential-evoked neuronal noradrenaline release and drug-induced

Table 1
Basal tritium outflow (b_1), electrically evoked tritium overflow (S_1) and S_2/S_1 ratios from SD-C and SD-MUN vessels.

	Basal outflow (b_1) (fractional rate of outflow; min^{-1})	Evoked overflow (S_1) (% of tissue tritium content)	S_2/S_1	n
Mesenteric artery				
SD-C	0.097 ± 0.009	0.221 ± 0.039	1.085 ± 0.091	7
SD-MUN	0.093 ± 0.007	0.348 ± 0.029*	1.076 ± 0.009	5
Mesentery vein				
SD-C	0.088 ± 0.006	0.265 ± 0.021	0.992 ± 0.057	7
SD-MUN	0.073 ± 0.006	0.381 ± 0.032*	1.078 ± 0.068	5
Tail artery				
SD-C	0.072 ± 0.007	0.163 ± 0.045	1.001 ± 0.098	7
SD-MUN	0.082 ± 0.009	0.268 ± 0.003*	1.086 ± 0.089	5

changes in evoked tritium overflow can be assumed to reflect changes in neuronal noradrenaline release, as observed in previous studies [22,26,27].

Electrical field stimulation (100 pulses/5 Hz) significantly increased tritium outflow from all vessels studied of both CONTROL and MUN rats (Fig. 1). The fractional rate of basal tritium outflow (b_1), electrically-evoked tritium overflow (S_1) of mesenteric arteries and veins and of tail arteries are shown in Table 1. Basal outflow and electrically-evoked tritium overflow remained constant throughout the CONTROL experiments, with b_n/b_1 and S_n/S_1 values close to unity. Electrically-evoked tritium overflow (S_1) was higher in MUN compared to CONTROL vessels (Table 1).

Tissue preparations of mesenteric and caudal arteries and mesenteric vein were pre-incubated with [^{3}H]-noradrenaline for 40 min. After pre-incubation with [^{3}H]-noradrenaline, tissues were superfused with [^{3}H]-noradrenaline free medium containing desipramine (400 nM). Tissues were stimulated twice at 30-min intervals (S_1-S_2; 100 pulses, 5 Hz, 1 ms, 50 mA): b_1 refers to the 5-min period immediately before S_1. The electrically-evoked tritium overflow was calculated by subtracting the estimated basal outflow from total outflow observed during and in the 25-min period subsequent to S_1 and expressed as a percentage of the tissue tritium content at the onset of stimulation. Values presented are means ± SEM and n denotes the number of tissue preparations. Significant differences of S_1 values from SD-C vessels: *P < 0.05.

3.3. Role of Ang II in vascular sympathetic neurotransmission

Ang II (30 nmol/L; a non-selective agonist of angiotensin receptors) facilitated electrically-evoked tritium overflow in all the vessels studied from both experimental groups, as shown by the ratio (S_2/S_1) in the absence of the drug. No statistical differences were observed between different vessels or between different experimental groups (Fig. 2), indicating that exogenous activation of angiotensin receptors is equivalent in arteries and veins and was not modified by maternal undernutrition.

Endogenous Ang II-mediated effects in vascular sympathetic neurotransmission were evaluated by blocking the AT1 receptor with a selective antagonist. In the presence of the AT1 receptor antagonist losartan (100 nmol/L), an inhibition of electrically-evoked tritium overflow was observed in all the MUN vessels studied (Fig. 2). Losartan was unable to modify tritium overflow in vessels from CONTROL rats. This finding is consistent with the occurrence of a tonic facilitation mediated by endogenous Ang II via AT1 receptor activation in MUN rats.

3.4. Localization of AT1 receptors adventitia mesenteric arteries

Adventitial layer of mesenteric arteries was identified from LSCM

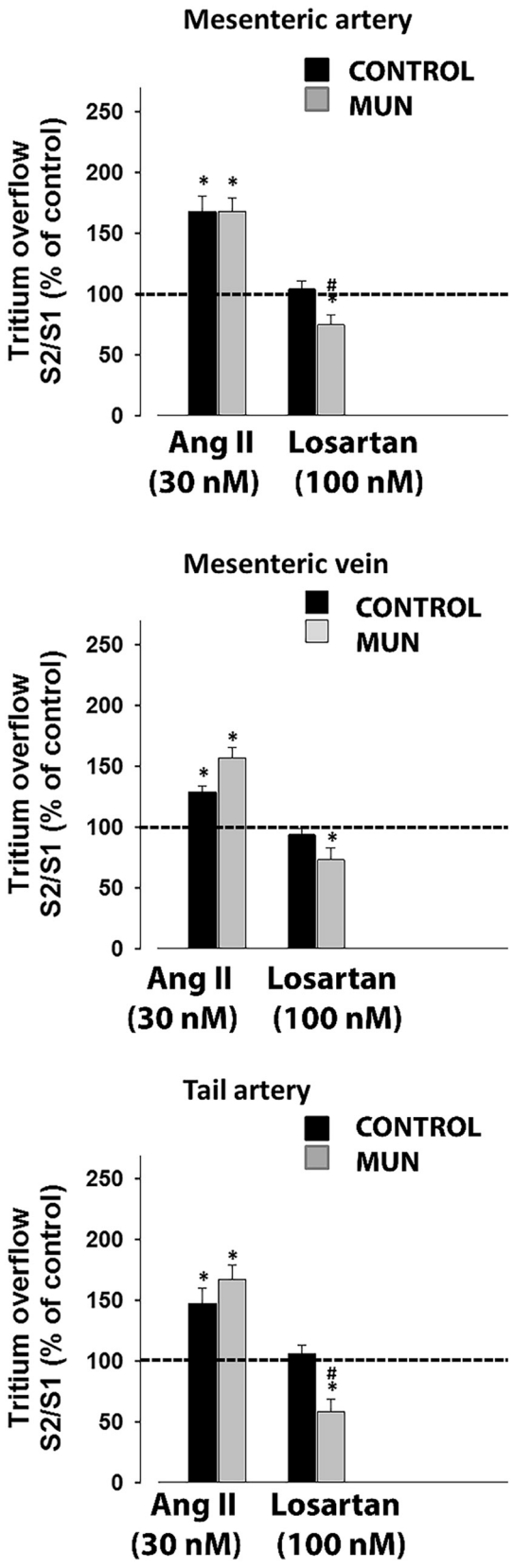

Fig. 2. Effects of Ang II (30 nM) or of losartan (100 nM) on the electrically-evoked tritium overflow from CONTROL and MUN vessels. Ang II was added 10 min before S_2 and was present until the end of the stimulation period. Losartan was added immediately after S_1 and kept until the end of the experiment. Ordinates: S_2/S_1 values obtained in individual tissue preparations, expressed as a percentage of the appropriate S_2/S_1 CONTROL value. Values are mean ± s.e.m. from 5 to 16 tissue preparations. Significant differences from the appropriate CONTROL: *P < 0.05; from the CONTROL vessels: #P < 0.05.

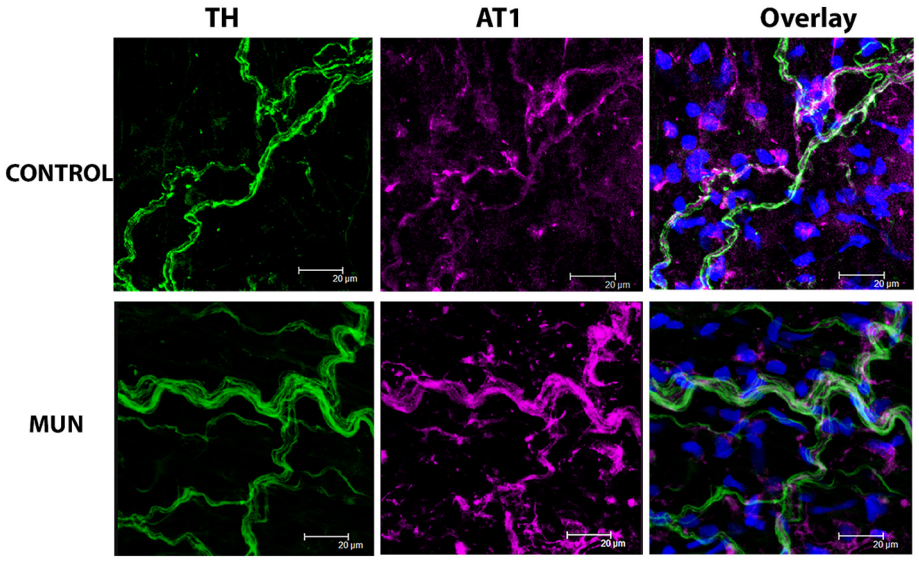

Fig. 3. Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries exhibiting TH (green), AT1 receptor (red), and overlay of AT1 receptor-TH immunoreactivities; nuclei are shown in blue. Scale bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

images by the shape and orientation of the nuclei [28]. No significant immunoreactivity was observed when the primary antibodies were omitted (negative controls; Supplementary material). Immunoreactivity for the sympathetic neuronal marker TH was evident in mesenteric arteries from both experimental groups (Fig. 3, green images). MUN vessels exhibited a larger immunoreactivity compared to CONTROL arteries.

LSCM also revealed the presence of AT1 immunoreactivity in both CONTROL and MUN mesenteric arteries (Fig. 3, red images). Quantitative analysis evidenced that the relative amount of AT1 immunoreactivity present in MUN arteries was much higher (up to 80% higher) than that exhibited by CONTROL vessels (Fig. 4A). These results together with functional data (Fig. 2) reveal and support differences in the sympathetic dynamic induced by AT1 receptors in MUN animals. It is relevant to notice the absence of a pattern compatible with a co-localization of TH with AT1 receptors immunoreactivities. This is in line with previous reports using this sympathetic neuronal marker and other membrane receptors [22,23] and, it is explained by the fact that AT1 receptors is present at the cytoplasmatic membrane while the enzyme tyrosine hydroxylase is storage inside vesicles in the sympathetic axoplasm [29]. Immunoreactivity for AT1 receptors show substantial overlay with TH marker suggesting that these receptors might be localized on the same structure, the postganglionic sympathetic nerves. Nonetheless, immunoreactivity for AT1 receptors in non-neuronal cells could also be observed.

Quantitative analysis of LSCM images revealed considerable differences in the relative mean of TH, AT1 and AT1-TH overlay between the two experimental animal groups (Fig. 4A): values obtained from MUN animals almost doubled comparatively to CONTROL.

Relative mean ratio of AT1 receptor and TH overlay, with respect to the total TH immunoreactivity, was slightly higher in MUN comparatively to CONTROL mesenteric arteries: 25% of sympathetic neurons exhibit AT1 receptor immunoreactivity in CONTROL versus 35% observed in MUN vessels (Fig. 4B). Also, the relative mean ratio of AT1 receptor and TH overlaid, regarding the total AT1 immunoreactivity revealed the presence of AT1 receptors in other cells than sympathetic neurons: 70% in CONTROL and 60% in MUN mesenteric arteries (Figs. 3 and 4C).

We have previously demonstrated that sympathetic nerves are surrounded by Schwann cells (anti-GFAP-immunoreactivity) [30]. The putative presence of AT1 receptors in Schwann cells was evaluated staining vessels with GFAP. Data from LSCM images evidenced the occurrence of AT1 and GFAP overlaid immunoreactivities (Fig. 5), confirming the presence AT1 receptors in adventitia cells other than the postganglionic sympathetic neurons.

4. Discussion

There is a gap in the knowledge regarding the complex interplay between vascular sympathetic neurotransmission and Ang II in FPH. This study points out that an increase vascular sympathetic activity might play an important role on hypertension development subsequent to fetal undernutrition. This is a relevant finding since sympathetic nervous system is a key contributor to vascular tone. Therefore, an increase in sympathetic neurotransmission would, likely, lead to an elevation in vascular resistance and subsequent elevation of blood pressure.

In MUN male rats a significant elevation in SBP was observed. This data in conscious rats is similar to that previously reported in male offspring from this rat model under anesthesia [31].

An increase in sympathetic neurotransmission was observed in our model of FPH both in arterial and venous territories. This data is in accordance with studies previously performed in a spontaneously hypertensive rat model, the SHR, where a hyper-sympathetic activity was also described in several arterial territories such as cerebral [32,33], tail [26] and mesenteric [26,27] and also in mesenteric vein [27]. Moreover, data is also in agreement with studies demonstrating that sympathetic nervous system activation seem to be increased in other models of FPH [34,35]. Some of these studies reported an increased circulating levels of noradrenaline: a model of intrauterine growth restriction induced by maternal protein restriction in the rat [36] and another model of placental insufficiency in both rat [37] and sheep [38,39]. In addition, chronic prenatal hypoxia is associated with hyperinnervation and an increase in adrenal medullary noradrenaline content and TH activity [40–42]. Therefore, and in accordance with those reported data, our findings indicate that the hyper-sympathetic activity observed in MUN vascular tissues can be explained by the larger amount of sympathetic innervation, as demonstrated by TH staining.

In MUN vessels, the observed increase in basal and electrically-induced noradrenaline release seems to be more evident in arteries than in veins. This data also suggests that the baseline tone is higher in arteries from MUN than in CONTROL. In fact, noradrenaline once released can activate α_1-adrenoceptors in vascular smooth muscle cells leading to vasoconstriction in arteries [43,44] and in veins [45,46] as noticed on splanchnic circulation and other vascular territories in animal models of hypertension.

We also observed that release of noradrenaline from sympathetic

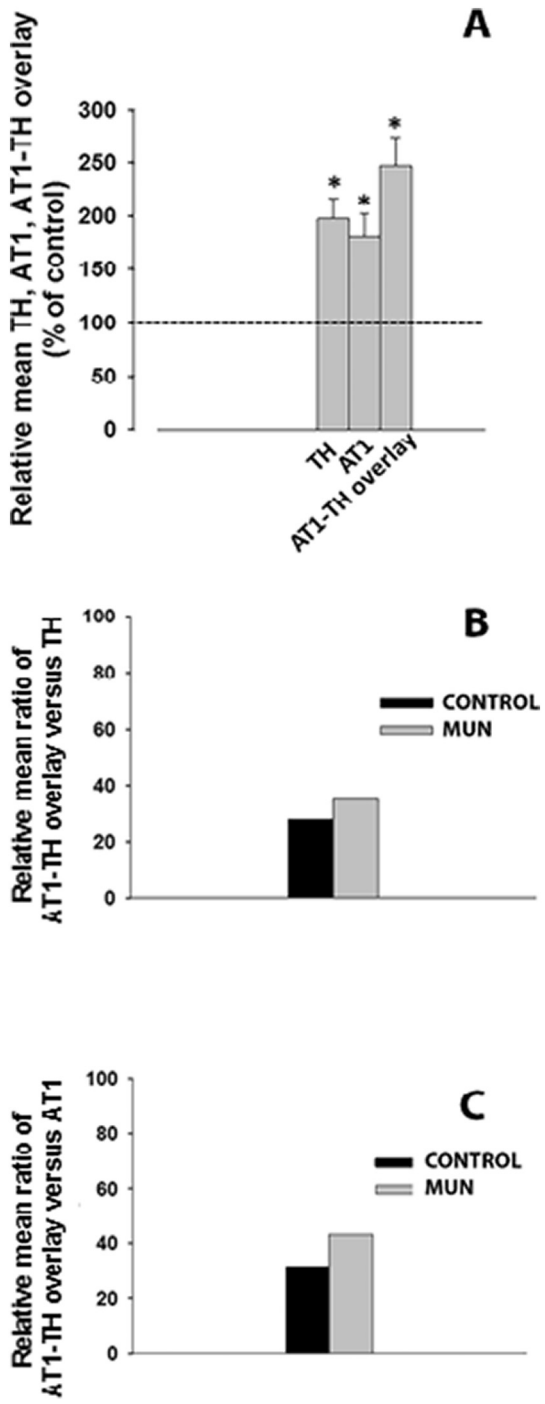

Fig. 4. Quantitative analysis of LSCM images from mesenteric arteries (CONTROL and MUN) staining with TH and AT1 antibodies. (A) Relative means of TH, AT1 and TH-AT1 overlay expressed as percentage of CONTROL values; (B) Mean percentage of overlay ratio with TH (C) and mean percentage of overlay ratio with AT1 are depicted. Images are reconstructions from 9 to 28 serial optical sections analyzed using PAQI software. Values are mean ± s.e.m., and n = 3–4 animals. Significant differences from SD-CONTROL: *P < 0.05.

nerve terminals was under the influence of Ang II through AT1 receptors. Thus, exogenous Ang II caused an increase in noradrenaline release both in arteries and veins from CONTROL and MUN groups, confirming the interplay between RAS and vascular neurotransmission. The facilitatory role of Ang II on noradrenaline release has been previously demonstrated in rat models of hypertension [47–50] other than FPH. In this study, the facilitation of noradrenaline release induced by exogenous Ang II was more pronounced in the arterial territories than in the veins from CONTROL animals. In addition, exogenous Ang II caused a similar increase in noradrenaline release in CONTROL and MUN but only in the arterial tissues. In the mesenteric veins, the facilitation of noradrenaline release was more marked in MUN than in CONTROL veins. These findings are in line with results obtained in other studies made in mesenteric veins of SHR animals [51].

It has been shown that inappropriate activation of the RAS may occur in response to a development insult: prenatal exposure to glucocorticoids up-regulates expression of the renal RAS [52]; central expression of the AT1 receptors is increased in offspring exposed to maternal protein restriction [53] and nicotine exposure [54]. However, in a maternal undernutrition animal model, these alterations had not been studied.

In our study, the treatment with AT1 receptor antagonist losartan was unable to modify tritium overflow in vessels from CONTROL rats. However, this procedure caused a facilitation in MUN vessels. This finding is consistent with the occurrence of a tonic facilitation mediated by endogenous Ang II via AT1 receptor activation. This effect was observed in all the MUN vessels studied.

Data support the possible occurrence of higher levels of endogenous Ang II in the sympathetic cleft of MUN rats. Curiously, the tonic effect, mediate by endogenous Ang II in prejuctional AT1 receptors was similar in all the MUN vessels studied, discarding the possibility that this vascular sympathetic alteration occurs only locally or in specific territories, but rather indicating a widespread effect both in resistance and capacitance vessels.

Taken together data revealed that local RAS is more effective in vascular sympathetic neurotransmission modulation in arterial territories than in veins. Furthermore, it also reinforces their relevance as a potential mechanism in FPH.

Since all the vessels studied showed a similar functional profile in vascular neurotransmission we chose the mesenteric artery as a representative vessel for the morphological study. LSCM data revealed the presence of nerve fibers (TH positive) both in CONTROL and MUN. We observed that sympathetic nerves spread through the adventitia reaching the medial layer. There was, however, a larger thickness of sympathetic nerve fibers in MUN mesenteric arteries accompanied by a parallel AT1 receptors overlaying these neurons. These higher amounts of AT1 receptors can explain, at least in part, the facilitatory tone regulating noradrenaline release from MUN mesenteric arteries stimulated with exogenous or endogenous Ang II. Data also show for the first time that AT1 are located in two main locations: one expressed in nerve fibers and another, in other adventitia cells. Some of these cells seem to be Schwann cells. This possibility was confirmed by data showing that AT1 receptors are overlaying GFAP, a marker for glial cells such as Schwann cells. In fact, the presence in Schwann cells, of other receptors or enzymes with a role on noradrenaline neurotransmission, was previously demonstrated [22,26] supporting a trophic role of these cells and of its close association with neurons. Images also show that these receptors are present in other type of adventitia cells like macrophages or mesenchymal cells. Altogether these data correlates well with the functional results obtained, showing an increase in the neuromodulatory role ascribed to Ang II in several vascular beds.

In summary, this study supports the occurrence of a hyper-sympathetic activation, involving a tonic facilitation, by endogenous Ang II, of prejunctional AT1 receptors, which can justify, at least in part, the occurrence of hypertension in these animals. These alterations also supports the possibility of vascular remodeling in MUN mesenteric arteries, as previously described in aorta [55]. A vascular structural alteration could also contribute to increase vascular resistance and the development of hypertension in this model of FPH.

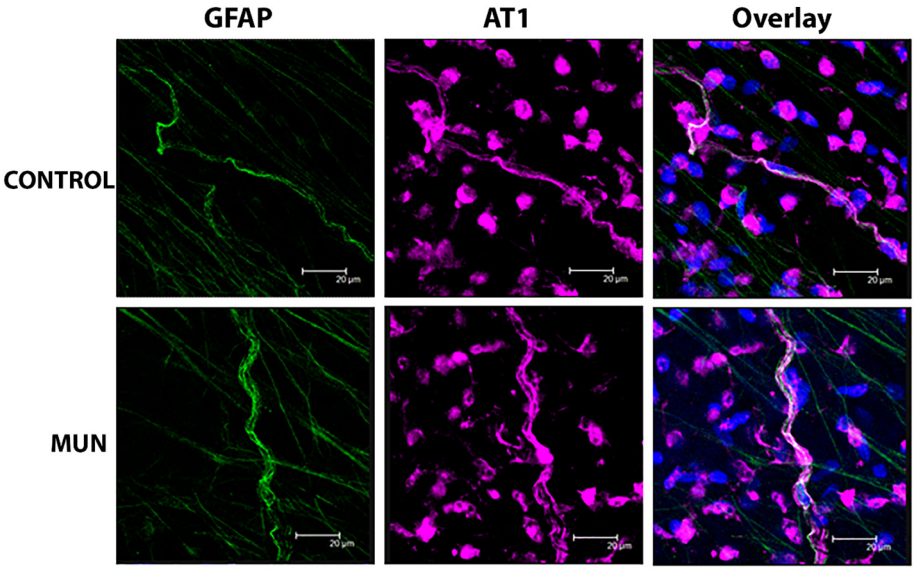

Fig. 5. Laser scanning confocal microscopy representative images of CONTROL and MUN mesenteric arteries exhibiting GFAP (green), AT1 receptor (red), and overlay of AT1-GFAP immunoreactivities, nuclei (blue). Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2018.10.003.

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Nuclear G-protein-coupled receptors as putative novel pharmacological targets

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Cell surface G-protein-coupled receptors (GPCRs) are targets for ~ 30% of drugs currently on the market, and are the largest group of gene products targeted by drugs. Until recently, signaling mediated by GPCRs was thought to emanate exclusively from the cell membrane as a response to extracellular stimuli. However, recent research has revealed the existence of nuclear (n)GPCRs with the ability to trigger identical and/or distinct signaling pathways to their respective counterparts on the cell surface. Understanding of the GPCR signaling platform on the nuclear membranes and its involvement in physiology and/or pathophysiology will be important to develop selective pharmacological and pharmaceutical approaches. In this review, we summarize our current understanding of nGPCRs, with emphasis on their potential as novel pharmacological targets.

Introduction

GPCRs belong to a diverse superfamily with ~ 800 members with heptahelical transmembrane domains [1]. According to the *IUPHAR Guide to Pharmacology*, GPCRs can be divided into six classes (A–F) based on their sequence homology: A (rhodopsin-like); B (secretin receptor family); C (metabotropic glutamate); D (fungal mating pheromone receptors); E (cyclic AMP receptors); and F (frizzled), with classes D and E not found in vertebrates [1].

Conventionally, GPCRs are cell surface receptors (mGPCRs) that respond to a variety of external stimuli through ligands including peptides or proteins, ions, lipids, odorants and light. GPCRs signal commonly through the heterotrimeric guanine (G) nucleotide-binding proteins (α, β, and γ subunits), mediating and dictating their downstream signaling and ultimate effects [2]. Over the past few decades, an increasing body of evidence has highlighted the existence of active GPCR signaling systems in intracellular compartments, including endosomes, endoplasmic reticulum (ER), mitochondria, and nucleus [3]. Some have been intensively studied, revealing attractive physiological functions and/or plausible roles in pathological conditions. Therefore, the occurrence of GPCRs other than mGPCRs requires a reassessment of, and development of new, models for pharmacological interventions. This is particularly important given that GPCRs are targets for approximately one-third of drugs used in clinical practice, acting on 108 GPCRs [4]. In this review, we provide a summary of the current knowledge regarding nGPCRs and discuss their potential as pharmacological targets for new and of already used drugs.

Nuclear GPCRs: from origin to function

In 1971, a pioneering study demonstrated that injection of angiotensin II (Ang II) in a nuclear zone induced ultrastructural cellular changes [5], an effect later attributed to the triggering of a GPCR located in the nucleus. Since then, evidence supporting the nuclear localization of GPCRs has been acquired mainly through the use of

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radioligand binding assays, electron and confocal microscopy techniques [immunolabeling, fluorescent ligands or receptors fused with fluorescent tag(s)], and western blots [6], using isolated nuclei and/or intact cells, and functional assays with caged and/or photolyzed ligands [7] or cytosolic microinjections [6].

More than 40 functional nGPCRs from several classes have been identified in the nuclear membrane of distinct cell types, as summarized in Table 1. Despite extensive research efforts, further clarification is required for some aspects of nGPCRs, including their exact nuclear location, relevant receptor and ligand origins, signaling cascades and ultimate effects. Nevertheless, nGPCRs appear to be evolutionarily conserved, given that their presence has been confirmed in a range of organisms, including nematodes (*Caenorhabditis elegans*), insects (*Drosophila*), and mammals, as also summarized in Table 1.

Origin of nuclear GPCRs

The appearance of GPCRs in the nucleus has been suggested to have occurred in at least four different ways (Fig. 1). nGPCRs could appear in the nucleus following mGPCR internalization and their subsequent translocation into the nuclear membrane [8]. Multiple mechanisms for mGPCR nuclear translocation (from cell membrane to nucleus) have been reported [8], via a process that can be either agonist dependent or independent. Agonist-dependent translocation of GPCRs occurs as a consequence of prolonged and/or repetitive exposure to agonists. In some cases, the receptor can be internalized along with the concomitant activation of signal pathways, such as the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) and/or phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways [9,10], which elicit genomic delay responses [11]. Once internalized, GPCRs can be recycled back to the cell membrane or transported to organelles such as lysosomes, ER, Golgi body [12–14], and mitochondria [3] or, ultimately, translocated into nucleus [3,11,15]. The GPCRs translocated from the cell membrane to the nucleus can (but not always) trigger a late response (e.g., gene transcription) which can complement (or oppose) the initial response triggered by mGPCR, without retaining the information obtained from the extracellular space.

Other stimuli or events can promote mGPCR nuclear import from the cell membrane via an agonist-independent process. Such GPCR activation challenges traditional GPCR pharmacology and introduces the environment as an important, and in some cases, decisive factor determining certain signal events and effects. For example, the neurokinin 3 (NK_3) receptor is activated, internalized, and transported to the nucleus following osmotic changes [16]. Other examples include the presence of parathyroid hormone 1 (PTH1) receptors in nuclear membranes of osteoblast-like cells only during certain stages of the cell cycle, when the DNA is exposed to transcriptional activity [17], and the presence of the vasoactive intestinal peptide 1 ($VPAC_1$) receptor, the translocation of which is induced by palmitoylation [18].

Agonist-dependent or -independent nuclear translocation appears to be cell type specific and depends on cellular conditions, such as concomitant activation with other receptors, metabolic state, or pathologies. The oxytocin (OT) receptor (OT [19]) and Ang II receptor type 1 (AT_1 [20,21]) have demonstrated both agonist-dependent and -independent nuclear localizing capabilities according to the cell type. Any alteration of any of the environmental conditions would lead to a modified response triggered by nGPCRs.

The translocation of GPCR to the nucleus has a specific fingerprint: in the C terminus or an intracellular loop of nGPCRs, short peptide sequences comprising basic amino acid residues (usually lysine/glycine-arginine repeats) have been found and are considered classical nuclear localization sequences (NLS) [22]. These NLS appear to direct GPCRs to the nucleus via small GTPases and/or importin mechanisms, as demonstrated for platelet-activating factor (PAF) [23], OT [11], protease-activated 2 (PAR2) [24], and PTH1 receptors [25].

The translocation of receptors might be independent on the presence of the NLS motive, as observed for the translocation of the bradykinin B_2 receptor, which, despite the NLS being present, is dependent on heterodimerization of the receptor with lamin C [26]. Furthermore, other peptide sequences that do not resemble classic NLS might also promote the nuclear import of receptors [27]. For example, the M9 sequence, a 38-amino acid-long fragment from heterogeneous nuclear ribonucleoproteins A1 and A2 proteins, is recognized by transportin, which mediates both nuclear import and export [28].

In addition to the cell membrane origin of nGPCRs, recent findings have suggested that resident nGPCRs are synthesized in ER (in an identical process to that described for mGPCRs) and traffic directly to the nucleus by lateral diffusion, as appears to occur with the endothelin subtype B (ET_B) receptor [29]. Other recent findings also suggest that GPCRs also appear in the nucleus through a fourth pathway: the direct synthesis of nGPCRs within the nucleus [30], a possibility supported by the occurrence of protein translation inside the nuclei of mammalian cells, supporting this as a possible direct source of nGPCRs [8,31].

Location and function of nuclear GPCRs

nGPCRs have been found in the nuclear envelope at the inner and/or outer nuclear membranes (INM and ONM, respectively) where they can be anchored securely (as demonstrated for the metabotropic glutamate 5 (mGlu5) receptor, which is firmly retained in the INM via its interactions with chromatin [32]) and oriented in such a way that exposes the effector-binding domain to either the nucleus or the cytosol (Fig. 2). nGPCRs have also been suggested to be present in the nucleoplasm in the network of invaginations of both nuclear membranes [33] or through transport within micelles, maintaining membrane-embedded GPCR [34]. However, the nuclear location of GPCRs does not suggest that nuclear signaling is limited to the nucleus. Evidence suggests the presence of receptors in the INM with the effector-binding domain directed towards the interior of nucleus, but with ultimate effects in the cytosol because of the translocation of effector proteins to the cytosol, as observed for the α_{1A}-adrenoceptor [35].

Key components of signaling pathways and the machinery typically associated with mGPCRs (G proteins, downstream effector molecules, second messengers, ion channels, and regulators) are found in the nucleus of several cell types [3,6]. These data reinforce the idea that the nucleus is an autonomous signaling organelle retaining functional nGPCRs. nGPCRs mainly regulate nuclear calcium $[Ca^{2+}]_n$, nitric oxide levels or cAMP production, although other second messengers, such as IP3, cGMP, and DAG,

TABLE 1

Nuclear GPCR subtypes in different cells and/or tissues and their respective functions

Family name	Subtype(s)	Cell/Tissue	Signalling/Function	Refs
Class A				
Adrenoceptors	α_1	Mouse cardiac myocytes	$G\alpha_q$–PLCβ1–ERK activation	[36]
	α_{1A}	Mouse cardiac myocytes	α_{1A}–$G\alpha_{q/11}$–PKCδ–"cTnI contractility"	[35]
	α_1, β_1	Rat ventricular myocytes		[6]
	β	Rat cardiomyocytes	$G\alpha_i$–PI3K–Akt–ERK1/2–↓ NF-κB transcription; ↑ Ripk2 + ↓ NF-κB, ATF-2, IL1r1 and Tnfrsf1b transcription	[22]
	β_3	Rat cardiomyocytes	$G\alpha_i$–NOS–GC–PKG transcription	[22]
	β_1, β_3	Rat and mouse ventricular cardiomyocytes	β_1–"AC–↑ cAMP transcription induction"; β_3–"$G\alpha_i$ transcription induction"	[6]
Angiotensin receptors		Rat hypothalamus, thalamus, septum, and midbrain		[56]
		Rat VECs, SMCs, and cardiomyocytes		[5]
		Rat liver and spleen		[57]
		Rat liver	↑ Renin + angiotensinogen transcription	[6]
		Rat and hamster myocytes	↑$[Ca^{2+}]_n$	[6]
	AT_1	Rat liver		[6]
		Rat and human VSMCs	↑ $[Ca^{2+}]_n$	[6]
		Human VSMCs	↑ $[Ca^{2+}]_n$	[6]
		Rat VSMC line A10	↑ p-CREB; ↑ proliferation	[6]
		Transfected HEK293 cells, mouse cardiac myocytes	↑ *COX2* gene transcription (PTGS-2)	[21]
		mRen(2).Lewis rat kidneys	↓ Nuclear AT_1	[51]
		Rat kidneys	PKC–NADPH oxidase (NOX4) activation; ↑ ROS	[70]
	AT_1, AT_2	Rat and mouse substantia nigra pars compacta (dopaminergic neurons), transfected MES 23.5 dopaminergic neuron cell line	AT_1–IP3–↑ $[Ca^{2+}]_n$–↑ AT_2 + angiotensin + PGC-1α + IGF-1 transcription → protective effect; AT_1–NOX4–↑ superoxide/H_2O_2; AT_2–NOS–↑ NO	[71]
		Rat liver	↑ Renin, angiotensinogen, c-myc, PDGF transcription	[6]
		Rat cardiomyocytes	AT_1/AT_2–↑ NF-κB expression; AT_1–"IP3–↑ $[Ca^{2+}]_n$ transcription"	[22]
		Sheep kidney	AT_2–↑ NO	[72]
		Sheep kidney of a fetal programming model	↑ ROS, NO	[73]
		Canine cardiac fibroblasts	AT_1–"IP3–↑ $[Ca^{2+}]_n$"; AT_2–↑ NO"; AT_1+AT_2 regulate fibroblast proliferation, collagen gene expression, and collagen secretion	[7]
Apelin receptor		Human cerebellum and hypothalamus, Purkinje cells and transfected D283 Med cells		[34]
Bradykinin receptors	B_2	Rat hepatocytes	↑$[Ca^{2+}]_n$–"Akt acetylation of histone H3–iNOS gene induction"	[22]
	B_2	Transfected HEK cells	Heterodimer with lamin C	[26]
	B_2	Rat olfactory bulb, cerebral cortex, hippocampus, basal forebrain, basal ganglia, thalamus, hypothalamus, cerebellum, and brainstem		[58]
	B_2	Human placenta		[74]
	B_2	HEK-293T cells		[34]
	B_1, B_2	Rat hippocampus		[59]
Chemokine receptors	CCR2	Transfected HEK cells		[15]
	CXCR4	Human non-small cell lung cancer		[52]
		Human hepatoma cancer cells		[6]
		Human prostate cancer cell lines	$G\alpha_i$–↑ $[Ca^{2+}]_n$	[22]
		HeLa cells		[6]
		Human nasopharyngeal carcinoma		[75]
Endothelin receptors		Rat liver		[6]
		Human endocardial endothelial cells and aortic VSMCs	↑$[Ca^{2+}]_n$–↑ ROS	[22]
		Rat heart	↑NO–↓ transcription	[76]
	ET_B	Human heart and VSMCs	↑$[Ca^{2+}]_n$	[6]
	ET_B	Rat cardiomyocytes	IP3–↑ $[Ca^{2+}]_n$	[29]
	ET_A, ET_B	Rat cardiac ventricular myocytes	↑$[Ca^{2+}]_n$	[42]
Formylpeptide receptors	FPR2	Human cancer cells	$G\alpha_i$–ERK2, c-Jun, and c-Myc phosphorylation	[22]
Ghrelin receptor		Transfected HEK293 cells		[77]

TABLE 1 (*Continued*)

Family name	Subtype(s)	Cell/Tissue	Signalling/Function	Refs
Gonadotrophin-releasing hormone receptors	$GnRH_1$	HEK 293 and HTR-8/Svneo cell lines	Histone H3 acetylation and phosphorylation	[22]
	Ce-GnRHR	*Caenorhabditis elegans*		[6]
Leukotriene receptors	$CysLT_1$	Human colon epithelial cells and colon cancer cells	Proliferative ERK1/2 signaling	[22]
		Human VSMCs	↑ $[Ca^{2+}]_n$–PAI-2 transcription	[22]
	$CysLT_1$, $CysLT_2$	Mouse B16, human HaCaT prekeratinocytes	NOX4 nuclear translocation, ROS accumulation, and oxidative DNA damage	[78]
Lysophospholipid receptors	LPA_1	Porcine cerebral microvascular endothelial cells	COX-2 and iNOS transcription	[22]
		Transfected HTC4 rat hepatoma cells		[6]
		Rat liver	$G\alpha_{i/o}$–PI3K–Akt–↑ $[Ca^{2+}]_n$/↑ iNOS expression	[6]
	LPA_1	Porcine cerebral endothelial cells	GC–eNOS/cGMP phosphorylation–↑ $[Ca^{2+}]_n$ + p42 MAPK activation, NF-kB binding to DNA + iNOS expression	[6]
	LPA_1	HBEC, PC12, and CHO cells	Regulates protein phosphorylation	[6]
	$S1P_1$	C57BL/6 mouse splenic CD4 T cells	$G\alpha_{i/o}$–↓ p-Erk + p-c-Jun	[46]
	$S1P_1$	Human umbilical vein endothelial cells	Cyr61 and CTGF expression	[79]
Melanocortin receptors	MC_2	H295R cell	Interaction with Nup50	[6]
Melatonin receptors	MT_2	Human placental choriocarcinoma cell lines		[6]
Muscarinic Acetylcholine Receptors		Rabbit cornea and endothelial cells	↑ cGMP, ↓ cAMP	[6]
		Chinese hamster ovary cells	↑ DNA and RNA polymerase II activity	[6]
Neuropeptide Y receptors		Rat pituitary gland		[6]
	Y_1	Human endocardial endothelial cells	↑ $[Ca^{2+}]_n$	[6]
Neurotensin receptor	NTS_1	Rat substantia nigra, transfected Chinese hamster ovary cells, and human lung cancer cell line		[80]
		Rat brain		[81]
Opioid receptors	**δ**	NG 108-I 5 neurohybrid cells		[6]
	μ	Human mesothelial cell line and human abdominal adhesions		[82]
	k	Mouse undifferentiated GTR1 cells	PKC–GATA-4 + Nkx-2.5 gene transcription	[83]
	k	Pluripotent embryonic P19 cell line	GATA-4 + Nkx-2.5 gene expression	[6]
	k	Hamster ventricular myocardial cells	PKC–opioid peptide gene transcription	[22]
Oxytocin receptors	OT	Mouse osteoblasts	Expression of osteoblast differentiation genes (*Sp7*, Atf4, *Ibsp*, and *Bglap*)	[11]
		Human osteosarcoma (U2OS, MG63, OS15, and SaOS2), breast cancer (MCF7), and primary human fibroblastic cells (HFF)		[19]
		Human epithelial glandular cells of endometrium, peritoneal endometriosis, and endometriotic ovarian cysts		[84]
Platelet-activating factor receptor	PAF	Rat liver	Phospholipase C–DAG	[6]
		Porcine cerebral microvascular endothelial cells and neurons	$G\alpha_{i/o}$–↓ cAMP; ↑$[Ca^{2+}]_n$; ERK1/2 + NF-**κ**B binding–iNOS + COX-2 transcription	[22]
		HEK293T and CHO-K1 cells and rat retinas	↑ Growth factors (e.g., VEGF) → angiogenesis	[23]
Prostanoid receptors	EP_1	Porcine cerebral microvascular endothelial cells		[6]
		Transfected HEK 293 cells		
		Murine fibroblast Swiss 3T3 cells (overexpressing EP1)	↑ $[Ca^{2+}]_n$–c-fos transcription	
		Pig myometrium	↑ $[Ca^{2+}]_n$–c-fos transcription	
	EP_1, EP_3	Rat cerebral cortex endothelial cells and neurons		
	EP_3	Piglet brain endothelial cells	$G\alpha_{i/o}$–K_{Ca2+} Channels–↑ $[Ca^{2+}]_n$–PI3K/Akt + Erk1/2 + NF-**κ**B–↑ eNOS expression	[22]
	EP_1, EP_2, EP_3, EP_4	Neonatal porcine brain		[22]
		Rat liver	EP_3–$G_{i/o}$–↑ $[Ca^{2+}]_n$	
		Porcine cerebral microvascular endothelial cells	↑ iNOS expression	
	TP	Rat oligodendrocytes	$G\alpha_s$ -↑cAMP–p-CREB + myelin basic protein transcription + ↑ survival	[39]
	TP	Rat oligodendrocytes progenitor cells	↑ Myelin basic protein expression	[85]

TABLE 1 (*Continued*)

Family name	Subtype(s)	Cell/Tissue	Signalling/Function	Refs
Proteinase-activated receptors/ coagulation factor II (thrombin) receptor-like 1	F2rl1/PAR2	Mouse retinal ganglion cells	Sp1 recruitment–↑ VEGFα expression → neovascularization	[47,24]
Tachykinin receptors	NK_1	Rat dorsal root ganglia		[6]
	NK_3	Rat ventral tegmental area		[6]
	NK_3	Rat hypothalamus		[16]
Urotensin receptor	(UT)	Rat brain	↑ Total transcription rate	[44]
		Monkey brain, human glioblastoma-astrocytoma U87-MG and human neuroblastoma SH-SY5Y cell lines		
		Rat and monkey heart (except right ventricle)	Transcription initiation	[38]
Class B				
Parathyroid hormone receptors	PTH1	Red deer osteoclasts		[6]
	PTH1	Mouse osteoblast-like cell line (MC3T3-E1)		[20]
	PTH1	Osteoblast-like cells (mouse MC3T3-E1, rat ROS 17/2.8 and UMR106, and human SaOS-2)	In MC3T3-E1 cells, receptors are involved in DNA synthesis and mitosis	[86]
	PTH1	Rat liver, kidney, uterus, gut, and ovary		[6]
VIP and PACAP receptors	VPAC	Human colonic adenocarcinoma cell line (HT29)		[6]
	$VPAC_1$	Human breast carcinoma cell lines (T47D and MDAMB-468)	$G\alpha_s$ –↑ cAMP	[22]
	$VPAC_1$	Chinese hamster ovary cells	Antiapoptotic activity	[18]
	$VPAC_1$, $VPAC_2$, PAC_1	Glioblastoma multiforme cell lines		[48]
Class C				
Metabotropic glutamate receptors	$mGlu_1$	Rat cortex, olfactory bulb, thalamus, and cerebellum, transfected HEK293 cells	↑ $[Ca^{2+}]_n$; control of brain development	[6]
	$mGlu_5$	Heterologous cells, mouse midbrain and cortical neurons, transfected HEK293 cells	↑ $[Ca^{2+}]_n$	[22]
		Rat striatal neurons	↑ $[Ca^{2+}]_n$ + p-CREB	[22]
		Rat and monkey substantia nigra		[87]
		Rat striatal neurons, HEK293 cells	$G\alpha_{q/11}$–PLC–IP3/ryanodine receptor –↑ $[Ca^{2+}]_n$	[6]
		Neonatal rat and mouse striatal cells	JNK, CaMK, CREB, ERK1/2 and Elk-1 phosphorylation; p-Elk-1–↑ c-fos + egr1	[6]
		Rat and mouse striatal neurons	$[Ca^{2+}]_n$ + ERK1/2 + CaMK–↑ cytoskeletal-associated proteins (Arc/Arg3.1)	[45]
		Rat hippocampus	↑ $[Ca^{2+}]_n$	[37]
		Rat spinal dorsal horn neurons	↑ $[Ca^{2+}]_n$; p-ERK1/2, Arc/Arg3.1 and c-fos → neuropathic pain	[50]
Orphans Class Frizzled	GPR158	Human trabecular meshwork cell line	Cell proliferation	[22]
	FZD_2 (C-terminal)	*Drosophila* muscle (neuromuscular junctions)	Promotes postsynaptic development of subsynaptic reticulum/postsynaptic membrane	[43,88]

Abbreviations: Akt, protein kinase B; ATF-2, activating transcription factor 2; $[Ca^{2+}]$n, nuclear calcium; CaMK, Ca2+/calmodulin-dependent protein kinase; COS-7, monkey kidney fibroblasts cell line; cTnI, cardiac troponin I; ERK, extracellular signal-regulated kinases; HEK, human embryonic kidney cells; HEK293, human embryonic kidney cells; IL1r1, interleukin 1 receptor, type I; IP3, inositol trisphosphate; NF-κB, factor nuclear kappa B; NOS, nitric oxide synthase; p-, phosphorylated; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; Ripk2, receptor-interacting serine/threonine-protein kinase 2; SMCs, smooth muscle cells; Tnfrsf1b, tumor necrosis factor receptor superfamily member 1B; VECs, vascular endothelial cells.

have also been described. These messengers influence several processes, including the redox status, transcription, cell proliferation, and tumorigenesis, as summarized in Table 1. However, whether nGPCR transduction mechanisms are similar to those described for mGPCRs needs to be clarified. nGPCRs have been studied with in situ microinjections or using isolated nuclei, allowing the identification of discriminative effects concerning the activation and signaling events triggered by nGPCRs versus mGPCRs. However, most of the methodologies used to study nGPCRs were developed for mGPCR studies (e.g., cell-permeable fluorescent dyes or appropriately targeted biosensors), resulting in limitations to targeting nGPCRs owing to their permeability and ability to discriminate between mGPCR and nGPCRs. Therefore, improved methodologies are required to obtain more discriminative information about the transduction mechanisms associated with nGPCRs.

nGPCRs can be constitutively active or activated by endogenous ligands. Extracellular ligands might reach nGPCRs through cellular uptake via selective transporters (e.g., noradrenaline [36]), through membrane exchangers (e.g., glutamate [37]), or via endocytosis through caveolin-coated pits (e.g., urotensin-II [38]). nGPCR exposure to ligands might differ from that observed for

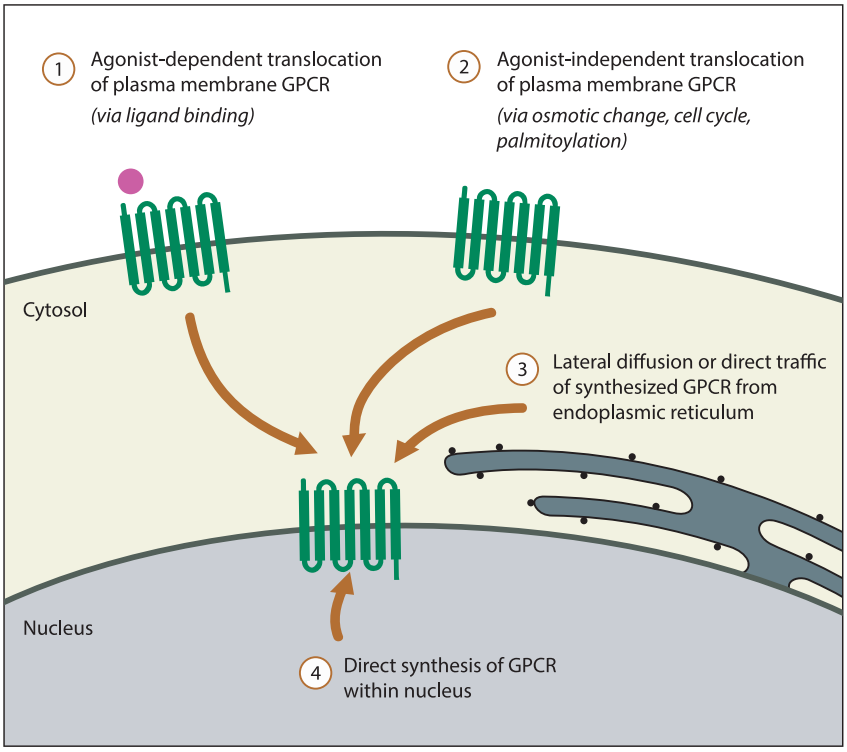

FIGURE 1

Schematic representation of conceivable origins for nuclear full-length G-protein-coupled receptors (GPCRs). Nuclear membranes might be an alternative destination for plasma membrane GPCRs that undergo nuclear translocation via an agonist-dependent (1) and/or -independent (2) pathway. Resident nuclear membranes GPCRs might be synthesized in the endoplasmic reticulum (ER) (3) or within the nucleus (4). (1) Receptor translocation from the cell membrane to the cell nucleus could occur as part of an agonist-mediated internalization event. Binding of an agonist to a receptor induces a first signaling phase at the cell membrane, which, after a long exposure, results in the desensitization and internalization of the receptors, culminating in nuclear translocation (frequently via small GTPases/importins mechanisms). (2) Other events and/or stimuli might also induce receptor internalization and nuclear translocation independently of agonist binding, such as palmitoylation or osmotic changes. (3) Nuclear import of newly rough ER-synthesized receptors can occur via lateral diffusion (given that the outer nuclear membrane is continuous with the ER) or from vesicular transport after ER and/or *trans*-Golgi network post-translational modifications. (4) Protein synthesis has been reported to occur in the nucleus, and is a plausible source of nuclear GPCRs.

mGPCRs because of the differential permeability of the extracellular ligands and of preferentially exposure to intracellular ligands that normally do not reach and influence mGPCRs. Indeed, nGPCRs might be preferentially activated by endogenous ligands that can be synthetized within the cell by enzymes located in the vicinity of the respective nGPCR, ensuring ligand bioavailability in the microenvironment of nuclear receptors. This type of process occurs for various ligands, including lipid mediators, such as prostanoids [39], platelet-activating factor, and lysophosphatidic acid [31], as well as peptides, namely apelin, bradykinin, Ang II [40], or endothelin [41]. All these attributes open new possibilities for determining pharmacological responses among these receptor subpopulations in future studies.

Nuclear GPCRs versus cell membrane GPCRs

The nuclear location of nGPCRs appears to be both cell type and tissue specific. Furthermore, the GPCR subtype has also been suggested as a feature contributing to differential nuclear versus cell membrane location. Different receptor densities have been observed at the cell and nuclear membranes, favoring one or other of the locations [34,42]: the nuclear membrane appears to be the preferential location for some GPCRs (e.g., ET_B [29]) whereas, for others, an alternative destination has been observed (e.g., AT_1 receptor [21]).

nGPCRs and mGPCRs usually present identical structures with a heptahelical chain sequence. However, there are exceptions, where only a small functional portion of the heptahelical chain might be translocated into the nuclear membrane, as reported for the Frizzled-2 (FZD_2) receptor, the C terminus of which is cleaved and then translocated into the nuclear membrane [43]. In some cases, GPCRs might undergo differential post-translational modifications between the cell and nuclear membranes [44]. For instance, the cell surface ET_B receptor undergoes *N*-glycosylation but this is absent in its nuclear counterpart [29].

Activation of nGPCRs can trigger responses aligned with those elicited by their respective cell surface counterparts. In this regard, similar signaling events have been described concerning the nuclear and cell surface mGlu5 receptors. Nuclear mGlu5 receptors have similar roles (i.e., induction of oscillatory Ca^{2+} responses) to those attributed to the cell surface mGlu5 receptors, with identical impacts on synaptic plasticity and the growth and/or differentiation of striatal neurons [45].

Interestingly, differential signaling between nGPCRs and mGPCRs has also been observed, such as in the case of sphingosine

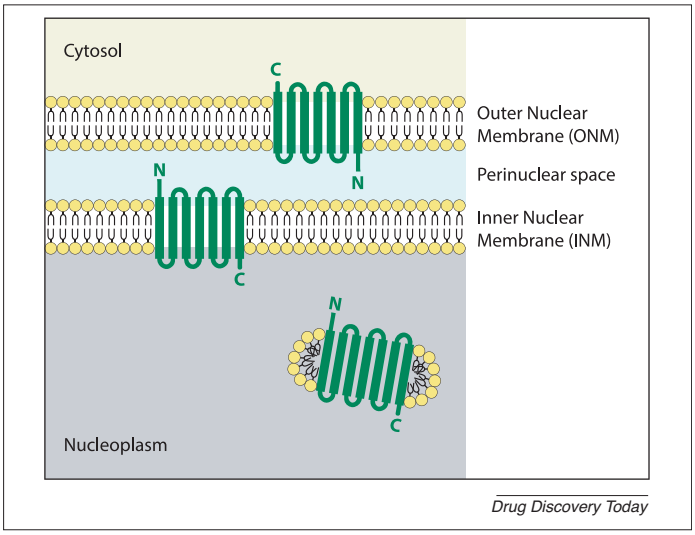

FIGURE 2

Schematic representation of the predicted topology of G-protein-coupled receptors (GPCRs) in the nucleus. Nuclear GPCRs were reported to be located at either nuclear envelope membranes (inner or outer) probably positioned in such a way that signals would be directed to the cytosol or nucleus. In some cases, nuclear GPCRs have been found within the nucleoplasm, possibly at the nuclear envelope invaginations or incorporated in micelles.

1-phosphate (S1P) subtype 1 ($S1P_1$) receptors. In unstimulated T cells, these receptors were not detected in the nucleus, whereas they were found to be involved in cell migration when located in the cell membrane. However, in stimulated T cells, nuclearization of most of the cell surface $S1P_1$ receptors appears to be induced and triggers a decrease in cell proliferation [46]. Another example of discriminated signaling events triggered by nGPCRs and cell surface counterparts is the PAR2 receptor: activation of the cell surface PAR2 receptor leads to expression of genes related to vessel maturation [47], whereas nuclear PAR2 receptor activation stimulates the recruitment of transcription factors that promote angiogenesis [47].

Nuclear GPCRs in pathological conditions

An increasing body of evidence has revealed that nGPCRs are involved in numerous key physiological and/or pathophysiological conditions, predominantly in the cardiovascular and nervous systems, but also in the reproductive system, liver, and kidney (Table 1). The density of nGPCRs also appears to be modified in pathological conditions. For example, nGPCR upregulation has been observed in cancer, pain and heart failure, whereas their downregulation was reported in hypertension. For instance, a higher density of nuclear VPAC1 receptors in high-grade gliomas was reported, possibly constituting a mechanism of tumor resistance [48]; cysteinyl leukotriene 1 ($CysLT_1$) receptors are present in the ONM, after internalization induced by prolonged exposure to leukotriene D4, and are upregulated in cancer [49]; in spinal dorsal horn neurons, ~80% of mGLU5 receptors are intracellularly located, mainly within nucleus (~60%), where they are functional active, and have recently been associated with persistent pain [50]; an increased density of AT_1 receptors was described in a heart failure model, associated with collagen gene expression and secretion and with the regulation of fibroblast proliferation [7]; the nuclear location of PTH1 receptors appears to be favored upon metabolic deprivation, whereas, under standard metabolic conditions, the cell surface and nuclear receptors are both present in similar amounts [25]. By contrast, in hypertension, the expression of nuclear AT_1 appears to be reduced in the kidney, probably as a compensatory mechanism because of increased levels of Ang II [51]. Nevertheless, further studies are needed to clarify the impact of nGPCRs in pathological conditions to evaluate their relevance as putative therapeutic targets.

Interestingly, the expression of nuclear C-X-C chemokine type 4 (CXCR4) receptors has been associated with significantly better outcomes in early-stage non-small cell lung cancer [52], suggesting that some nGPCRs could be used as predictors of disease outcome. It is also conceivable that, after the improvement and validation of methodologies that allow refined discrimination between effects ascribed to different GPCRs populations, nGPCRs could be used to monitor the evolution of disease.

Are nuclear GPCRs pharmacologically targetable?

The existence of GPCRs in the nucleus opens new opportunities and challenges for pharmacological intervention using these targets. GPCRs are targets of approximately one-third of drugs currently used in the clinic, acting on >100 types of receptors, and 94% of them are class A GPCRs [4]. More than 80% of these drugs target GPCRs such as aminergic receptors (adrenoceptors, muscarinic acetylcholine, histamine, dopamine, and 5-hydroxytryptamine receptors) and opioid receptors (gpcrdb.org) signaling mostly via the $G\alpha_{i/o}$ (~38%), $G\alpha_{q/11}$ (~29%), and $G\alpha_s$ (~24%) subunits [53]. Therefore, it is plausible that some of the effects of these drugs result from actions on nGPCRs. For example, activation of α_{1A}-adrenoceptors promotes cell cycle arrest, hypertrophy, and the differentiation of rat-1 fibroblasts into smooth muscle cells as well as the expression of negative cell cycle regulators by a mechanism independent of the cAMP/PKA signaling pathway [54]. Although not addressed, the involvement of nuclear adrenoceptors might explain these effects. Thus, the possibility that nuclear α_{1A}-adrenoceptors contribute to the clinical effects of α_{1A}-adrenoceptor antagonists used in the treatment of benign prostatic hyperplasia cannot be discarded. The same rationale can be applied to $CysLT_1$-selective antagonists, such as montelukast, used in the treatment of asthma, or to AT_1 receptor antagonists, such as losartan, used in hypertension and heart failure, where the reported presence of the respective receptors in the nuclear membranes might partially contribute to their clinical efficacy.

In addition to drugs that interact directly with receptors, several others might influence the intracellular availability of ligands indirectly and, possibly, the ligand concentration in the microenvironment of nuclear receptors. The most obvious example is zileuton (a 5′-lipoxygenase inhibitor used in the treatment of asthma; 5′-lipoxygenase is an enzyme involved in the initial events of leukotriene synthesis, which occurs at the nuclear membrane [55]): when 5′-lipoxygenase is inhibited by zileuton, it impacts the concentration of leukotrienes available for nuclear leukotriene receptors. Other examples of drugs the therapeutic effects of which might have a contribution from nuclear GPCRs include captopril and other inhibitors of angiotensin-converting enzymes. These drugs interfere simultaneously with two endogenous ligands (a decrease in Ang II and the accumulation of bradykinin), the

receptors of which (AT_1 and AT_2 receptors [6,7,22,56,57] and bradykinin B_1 and B_2 receptors [58,59], respectively) were reported to have a nuclear location. In terms of adrenoceptors, several drugs are envisaged to have the potential to interfere with the concentration of their endogenous ligands (noradrenaline and adrenaline), namely monoaminoxidase (MAO) inhibitors, reserpine, and amphetamines. MAO inhibitors have been used in the treatment of depression and Parkinson's disease, and their effects have been explained by sparing monoamines (such as noradrenaline, adrenaline, dopamine, histamine, and serotonin) from metabolism and, consequently, favoring their cytosolic accumulation in the synaptic vesicles. However, such accumulation can also impact the ability of these monoamines to reach other intracellular targets, namely nGPCRs. Therefore, the involvement of nGPCRs could explain the alterations in gene expression caused by MAO inhibitors [60,61]. nGPCRs might also have a role in the effects mediated by reserpine. Reserpine, an antipsychotic drug (also used in hypertension), inhibits the vesicular ATP/Mg^{2+} pump, leading to a cytosolic increase in monoamines that might target nuclear adrenoceptors. This could explain the increase in gene transcription observed after reserpine administration [62,63]. The involvement of nGPCR-mediated effects is also a possibility in the case of recreational drugs with a high risk of addiction, such as amphetamines: the stimulatory effects of amphetamines result from the transient facilitation of neurotransmission, which is caused by monoamine transport from the cell, instead of the usual transport into cell [64]. However, addiction to these drugs is coupled with long-lasting transcriptional alterations that require alternative explanations [65,66]. The possibility that they result from a reduction in the number of messengers available intracellularly to activate nGPCRs is a tempting hypothesis.

The development of new drugs with higher affinity toward nGPCRs than to their cell surface counterparts is a challenging field of research. Therefore, an improved understanding of the different signaling events triggered by nGPCRs and their cell surface counterparts will be relevant to enable the development of drugs targeting solely nGPCRs. Drugs with preferable activity on nGPCR membrane receptors should be designed using techniques such as molecule modification through the addition of a 'caging' moiety, a removable functional group that increases cell permeability and decreases affinity for the cell surface receptor [67]. This type of approach could be sufficient to ensure the activation of nGPCRs, although without discriminative properties that could rule out the activation of mGPCRs on the cell surface. Other approaches could be the use of nanoparticles [68] or cell-penetrating peptides [69] that enter cells usually by endocytosis and/or can be coupled with a NLS peptide, taking advantage of the natural active transport system of the cell into the nucleus. The use of 'Trojan Horses' (i.e., drugs inside a carrier that is sensitive to intracellular enzymes) could be a strategy to circumvent the activation of cell membrane receptors and to deliver drugs preferentially into the intracellular environment, providing more possibilities to be translated into clinical practice.

Concluding remarks

GPCRs were thought to solely convert extracellular stimuli into intracellular responses, while being located exclusively on cell surface. However, over the past few decades, the intracellular location of GPCRs in nuclear membranes was verified, challenging the classic view of GPCRs. nGPCRs might be specifically or additionally activated with overlapping and/or unique signaling events comparative to those of their cell surface counterparts. It has been hypothesized that the final downstream effects attributed to a certain GPCR might result from the integration of extracellular and intracellular signaling pathways.

Studies thus far have led to increased insights into the involvement of nGPCRs and their differential signaling in key physiological and pathophysiological processes. In addition, the density of some nGPCRs is known to be increased in several pathological conditions, highlighting them as putative therapeutic drug targets. Therefore, the nuclear location of GPCRs challenges our traditional understanding of GPCR pharmacology and exponentially increases their complexity. Comprehensive pharmacological studies are urgently needed for the development of new optimized drugs with activity on a desired intracellular response to reach the directed therapeutic outcome. In addition, further studies are required to understand and challenge the classic pharmacological view of drugs used in clinical practice the mechanisms of action of which have been attributed so far solely to GPCRs located in the cell membrane. Thus, our new understanding highlights the complex diversity of GPCR pharmacology, requiring the inclusion of different locations and/or functions for GPCRs in physiopathology and disease.

Conflicts of interest

The authors report no conflicts of interest.

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